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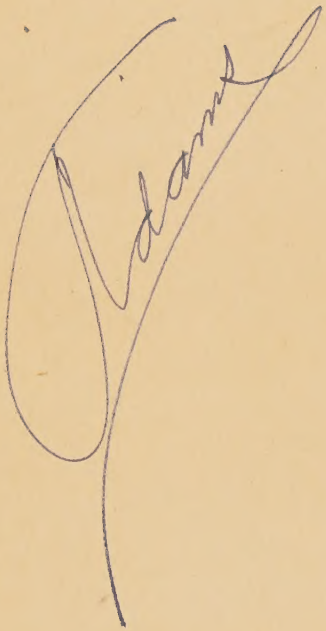

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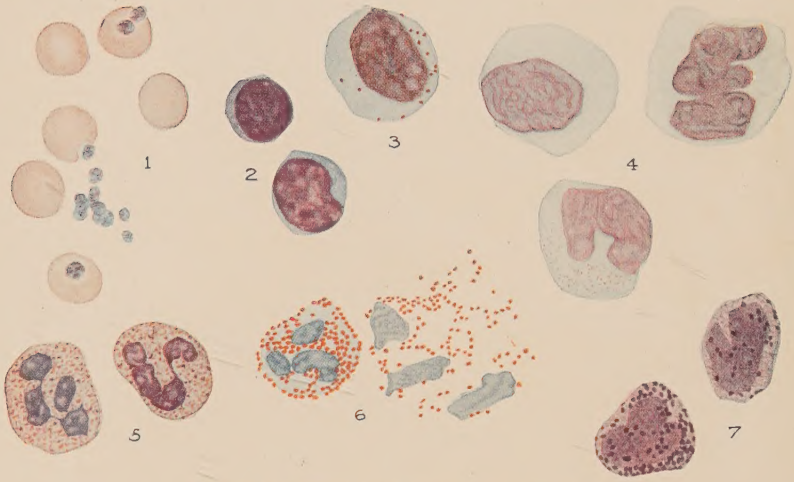
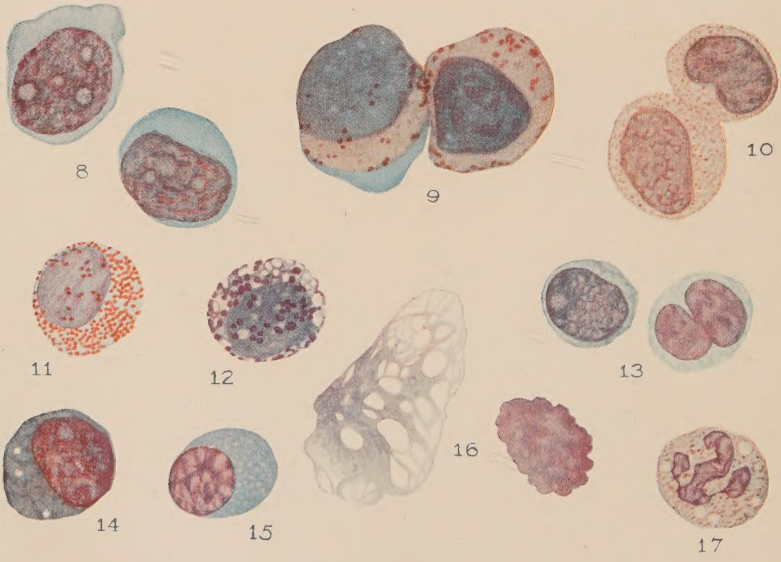
PLATE I



The cells of normal blood (scale: 1 mm. = 1 μ).



Leukocytes which appear in the blood in disease (scale: 1 mm. = 1 μ).

(J. W. Rennell, pinx.)

Explanation of Plate I

Fig. 1.—The cells of normal blood reproduced from actual cells. Wright's stain (× 1000: 1 mm. = 1 μ).

1, Red corpuscles and blood-platelets. 2, Two lymphocytes. 3, A lymphocyte with azurophilic granules. This cell lay in a thin portion of a film and was exceptionally large. 4, Three endothelial leukocytes, one with fine cytoplasmic granules. The granules are rarely so distinct as here shown. 5, Polymorphonuclear neutrophils. 6, Eosinophils, one ruptured. The cells selected for drawing contained fewer granules than are usual. 7, Basophils.

Fig. 2.—Leukocytes found in the blood in disease. All reproduced from actual cells stained with Wright's stain, excepting No. 15, which is copied from Pappenheim (× 1000 : 1 mm. = 1 μ).

8, Two myeloblasts, showing nucleoli. 9, Two young myelocytes. Note the blue edge of one. 10, Two mature neutrophilic myelocytes. 11, Eosinophilic myelocyte. 12, Basophilic myelocyte. Some of the granules have dissolved, leaving vacuoles and staining the cytoplasm. 13, Two lymphoblasts, one with lobulated nucleus (Rieder cell). 14, Turck's irritation leukocyte with vacuoles. 15, Plasma cell. 16, Degenerated nuclei, one a so-called "basket cell." 17, Neutrophilic leukocyte with vacuoles.

CLINICAL DIAGNOSIS
BY LABORATORY METHODS
A WORKING MANUAL OF CLINICAL PATHOLOGY

By

JAMES CAMPBELL TODD, PH. B., M. D.

Professor of Clinical Pathology, University of Colorado School of Medicine

and

ARTHUR HAWLEY SANFORD, A. M., M. D.

Professor of Clinical Pathology, University of Minnesota (The Mayo Foundation);
Head of Section on Clinical Laboratories, Mayo Clinic

SIXTH EDITION, REVISED AND RESET
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FOREWORD

It is now almost twenty years since the appearance of the first edition of this book. It had had its origin some three years earlier as a set of notes written for my lectures upon clinical pathology, a subject which, at that time, was beginning in a few schools to receive some attention as worthy of a short course in the medical curriculum, usually under the name "clinical diagnosis" or "medical microscopy." The subject matter was very limited, comprising little more than elementary microscopy, blood-counts, and a few simple tests which any physician could readily carry out in his office.

Since that time clinical pathology has undergone marvelous development, and the book has of necessity grown in size and scope with each new edition. The direction and extent of its growth have naturally been determined by the growing needs of my students, as these became apparent through discussions which arose in the classroom and through the difficulties which the students encountered in attempting to carry out and interpret the various procedures in the laboratory. The steadily increasing favor with which the successive editions have been received by students, teachers, and laboratory workers generally has encouraged me to believe that the book has indeed met the needs of those for whom it was intended, and has been most gratifying.

The repeated and thoroughgoing revisions have been a congenial task; but they have also been laborious, and ill health has now made it seem advisable to find some one to divide the work with me. In this situation I have been exceedingly fortunate in enlisting the collaboration of so able a man as Dr. Arthur H. Sanford, whose broad interest in clinical pathology and whose long experience in the Mayo Clinic and the Graduate School of the University of Minnesota are well known. Dr. Sanford has consented to join me as co-author, and we are now equally responsible for the book.

I wish to take this opportunity to express to the publishers, W. B. Saunders Company, my sincere appreciation of their liberal publication policy which has put no restrictions on the book or its illustrations, and of their exceptional and uniform courtesy throughout the years of our association.

James C. Todd.

University of Colorado,
Boulder, Colorado.

PREFACE TO THE SIXTH EDITION

In the present edition, while the scope of this book has not been materially changed, its size has been considerably increased, and its form slightly altered. It is hoped that its value has thereby been enhanced without sacrifice of the simplicity and conciseness which were its original aim. As before, chief emphasis has been laid on methods and microscopic morphology.

Much of the new material is the outgrowth of questions and discussions which have arisen in classroom and laboratory. To one who sees much of the work of students in the clinical laboratory, it soon becomes evident that errors in microscopic diagnosis spring less frequently from ignorance of the typical appearance of microscopic structures than from imperfect preparation of the material, faulty manipulation of the microscope, or failure to recognize extraneous structures, artifacts, and various misleading appearances. Such sources of error have been given especial attention.

Each chapter has been carefully revised in the light of the numerous advances in clinical pathology since the last edition was prepared, and some sections have been rearranged or rewritten. The changes and additions are widely scattered throughout the book, hence most of them evade particular mention. A number of the newer, well-tried methods in blood chemistry have been added. Rosenthal's newest technic for the liver-function test is described. The latest revision of Kahn's flocculation test for syphilis is included. Perhaps the most complete revision has been in the chapter on Animal Parasites, which has been largely rewritten. New material has been added in the chapter on Bacteriology, and there is recognition of the new classification proposed by the Committee of the Society of American Bacteriologists on characterization and classification of bacterial types. A few methods which are little used have been omitted, while the discussion of others has been somewhat abbreviated.

In a book which deals largely with clinical microscopy accurate pictures of microscopic structures should play a large if not predominant part. They give information which cannot be conveyed in any other way. There has been a careful revision of the illustrations; the poorer ones have been omitted or replaced with those that are more desirable; 35 new illustrations appear in this edition. Most of the illustrations were made from material used for teaching purposes, or found in routine diagnostic laboratory work. Many of these are photomicrographs by the authors. Inadequate as is the photomicrograph in some fields, its value in clinical microscopy cannot be questioned.

As before, the names of those to whom credit is due for material used are given in the text, but often the references to places of publication are not included. Given the names of the authors, such information can easily be found in the *Index Medicus* or the *Quarterly Cumulative Index of Medical Literature.* A moderate number of references to articles in recent periodicals has, however, been included as suggestions for supplementary reading.

We wish to express our appreciation to all who have helped with the making of the present edition, and especially to Dr. Mugrage and Dr. Beacom of the University of Colorado, and to Dr. Magath of the Mayo Clinic.

James C. Todd,
Arthur H. Sanford.

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CLINICAL DIAGNOSIS

BY LABORATORY METHODS

INTRODUCTION

USE OF THE MICROSCOPE

THERE is probably no laboratory instrument whose usefulness depends so much upon proper manipulation as the microscope, and none is so frequently misused by beginners. Some suggestions as to its proper use are, therefore, given at this place. It is presumed that the reader is already familiar with its general construction (Fig. 1).

For those who wish to understand the principles of the microscope and its manipulation—and best results are impossible without such an understanding—a careful study of some standard work upon microscopy, such as those of Simon Gage, E. A. Spitta, and A. E. Wright, is earnestly recommended. It is also recommended that the beginner provide himself with some slides of diatoms, for example, *Pleurosigma angulatum*, *Surirella gemma*, and *Amphipleura pellucida*, costing fifty cents each, and with some good preparations of stained and unstained blood. The blood slides can easily be made from one's own blood, as described in Chapter III. Faithful practice upon such test-objects, in the light of the principles of microscopy, will enable the student to reach, intelligently, an accuracy in manipulation to which the ordinary laboratory worker attains only slowly and by rule of thumb. He will soon find that the bringing of an object into accurate focus is by no means all of microscopy.

Source of Light.—Good work cannot be done without proper illumination, and this is, therefore, the first and most important consideration for one who wishes to use the microscope effectively.

The light which is generally recommended as best is that from a white cloud, the microscope being placed by preference at a north window, to avoid direct sunlight. At any other window a white window-shade is desirable. Such light is satisfactory for all ordinary work. Artificial light is, however, imperative for those who must work at night, and is a great convenience at all times. Properly regulated artificial light, moreover, offers decided advantages over daylight for critical work. Almost any strong light which is diffused through a frosted globe will give fair results. The inverted Welsbach light with such a globe is excellent, as is also the Mazda incandescent lamp with a frosted bulb. Such a bulb may conveniently be inclosed within a tin or paste-board box, with small openings in the back for ventilation and a circular window in the front to transmit the light. At the University of Colorado, where the students do much of their microscopic work by artificial light, the inexpensive lamp shown in Figure 2 was at one time very popular. It has the advantage that the eyes are shaded from the glare, while at the same time there is abundant light for drawing or writing upon the table beside the microscope. All such lights have a yellow tinge, and to counteract this a blue glass disk, usually supplied with the microscope, is placed in a supporting ring beneath the condenser. The following plan is much used abroad, and gives results equal to the best daylight: A Welsbach lamp or strong electric light is used, and a spheric glass globe—a 6-inch round-bottom flask answers ad-

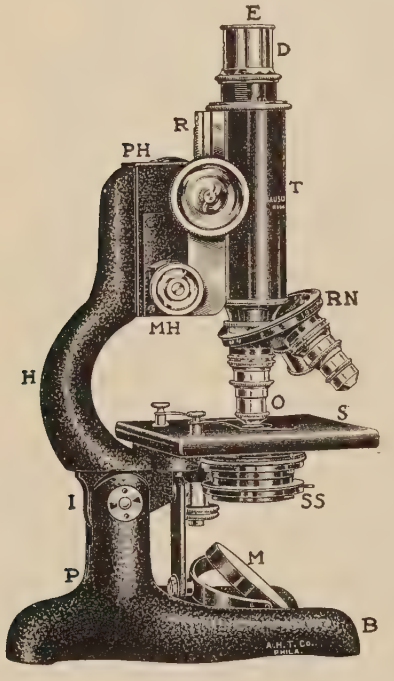


FIG. 1.—A satisfactory type of microscope for medical work: *E*, Eye-piece; *D*, draw-tube; *T*, body-tube; *RN*, revolving nose-piece; *O*, objective; *R*, rack; *PH*, pinion head for coarse focusing; *MH*, micrometer head for fine focusing; *H*, handle; *S*, stage; *SS*, substage; *M*, mirror; *I*, inclination point; *P*, pillar; *B*, base.

mirably—is placed between it and the microscope, to act as a condenser (Fig. 3). The flask should be at a distance equal to its diameter from both the light and the mirror of the microscope.

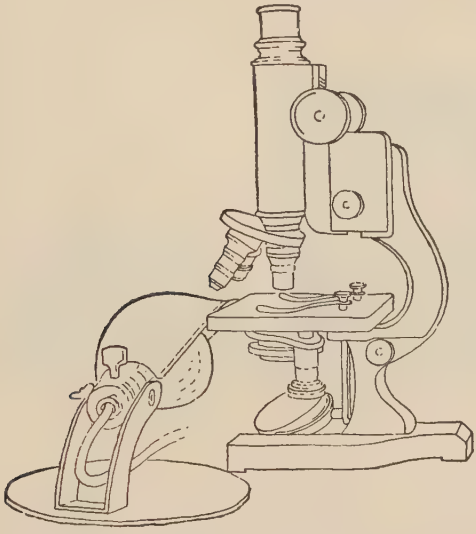


FIG. 2.—A convenient lamp for use with the microscope. It should be placed close to the microscope so that the shade protects the eyes from the glare. A blue glass disk is placed in the ring beneath the condenser.

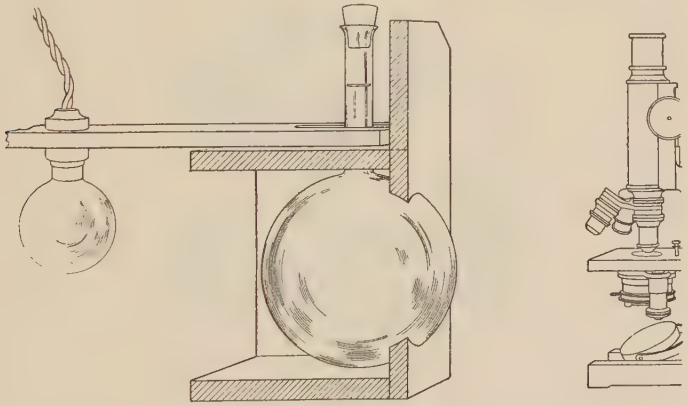


FIG. 3.—Illumination with water-bottle condenser. One side of the wooden box has been removed to show the construction.

In order to filter out the yellow rays the flask is filled with water, to which have been added a few crystals of copper sulphate and a little ammonia.

Within the past few years manufacturers have paid more attention than formerly to means of artificial illumination, and most of them now offer several types of lamp. Two good types are shown in Figures 4 and 5. Both can be fitted with light filters made of the newly invented "daylight glass," which, when used

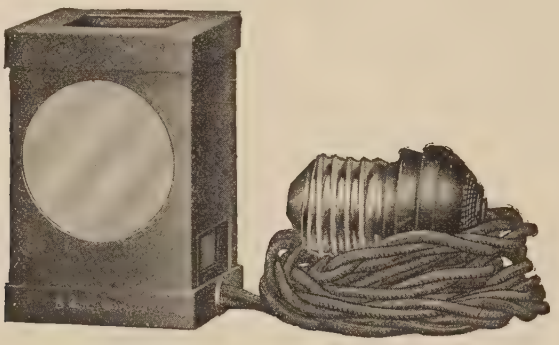


FIG. 4.—Small microscope lamp with daylight-glass filter.

with the nitrogen-filled tungsten lamp, transmits a light practically indistinguishable from daylight either visually or spectrophotometrically.

The microscope lamp should not stand at so great a distance from the microscope that its image fails to fill the aperture of the

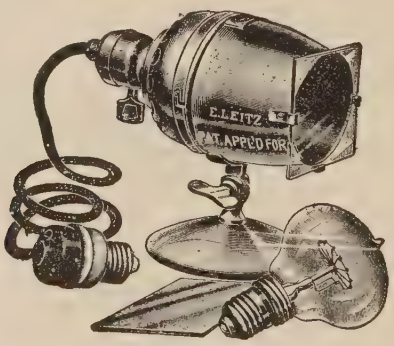


FIG. 5.—An excellent type of microscope lamp suitable both for ordinary work and for dark-field illumination.

condenser—a condition which one can readily detect by removing the ocular and looking down the tube.

Forms of Illumination.—After one has arranged the microscope in proper relation to the source of light, whether this be daylight or any of the artificial sources mentioned above, the next

problem is to secure an evenly illuminated field of view without mottling or any trace of shadows. This is accomplished by manipulating the mirror and the condenser. Following this, the direction and the amount of light must be considered in relation to the character of the object under examination as is indicated in the following paragraphs.

Illumination may be either *central* or *oblique*, depending upon the direction in which the light enters the microscope. To obtain **central illumination** the mirror should be so adjusted that the light from the source selected is reflected directly up the tube of the microscope. This is easily done by removing the eye-piece and looking down the tube while adjusting the mirror. The eye-piece is then replaced, and the light reduced as much as desired by means of the diaphragm.

Oblique illumination is obtained in the more simple instruments by swinging the mirror to one side, so that the light enters the microscope obliquely. The more complicated instruments obtain it by means of a rack and pinion, which moves the diaphragm laterally. Beginners frequently use oblique illumination without recognizing it, and are thereby much confused. If the light be oblique, an object in the center of the field will appear to sway from side to side when the fine adjustment is turned back and forth.

The **amount of light** admitted is also imporant. It is regulated by the diaphragm.

The bulk of routine work is done with central illumination, and, therefore, every examination should begin with it. Each of the forms of illumination, however—central and oblique, subdued and strong—has its special uses and demands some consideration here. The well-known rule, "Use the least light which will show the object well," is good, but it does not go far enough.

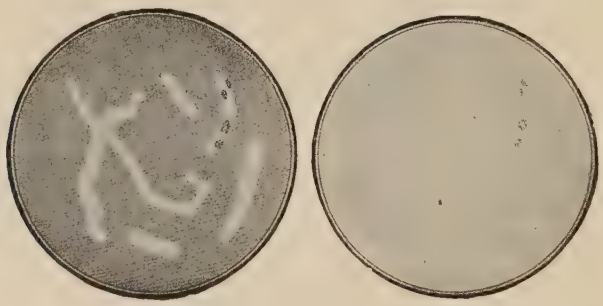
In studying any microscopic structure one considers: (1) its color, (2) its outline, and (3) its surface contour. No one form of illumination shows all of these to the best advantage. It may, therefore, be necessary to change the illumination many times during a microscopic examination.

To see color best, use central illumination with strong light. The principle is that by which a stained glass window shows the purest color when the light is streaming through it. Strong central light is, therefore, to be used for structures such as stained bacteria,

whose recognition depends chiefly upon their color, and, alternating with other forms, for stained structures in general.

To study the outline of an object use very subdued central illumination. The diaphragm is closed to the point which trial shows to be best in each case. This illumination is required by delicate colorless objects, such as hyaline tube-casts and cholesterol crystals, which are recognized chiefly by their outline. The usual mistake of beginners is to work with the diaphragm too wide open. Strong light will often render semitransparent structures entirely invisible (Fig. 6).

To study surface contour use oblique light of a strength suited to the color or opacity of the object. In routine work oblique illumination is resorted to only to study more fully some object which has been found with central illumination, as, for instance, to demonstrate the cylindric shape of a hyaline tube-cast.



A B

FIG. 6.—A, Hyaline casts, one containing renal cells; properly subdued illumination; B, same as A; strong illumination. The casts are lost in the glare, and only the renal cells are seen. (From Greene's "Medical Diagnosis.")

Dark-field illumination consists in blocking out the central rays of light and directing the peripheral rays against the microscopic object from the side. Only those rays which strike the object and are reflected upward pass into the objective. The object thus appears bright upon a black background. By means of this form of illumination very minute structures can be seen, just as particles of dust in the atmosphere become visible when a ray of sunlight enters a darkened room.

Dark-field illumination for low-power work can be obtained by means of the ring stops with central disks which accompany most microscopes when purchased. The stop is placed in a special ring beneath the condenser. When the regular stop is not at hand, one

can use the glass disk which is generally supplied with the microscope or an extra large round cover-glass, in the center of which is pasted a circular disk of black paper. The size of the black disk depends upon the aperture of the objective with which it is to be used, and can be ascertained by trial. For best results the condenser should be oiled to the under surface of the slide and should be focused on the object under examination.

For oil-immersion work a special condenser is necessary. This is sold under the name of reflecting condenser or dark-field illuminator. In its most desirable form this is interchangeable with the regular substage condenser. One type has a lamp built in or attached beneath it (Fig. 7). Some makers now offer a condenser which allows one to change almost instantly from bright-field to dark-field, and vice versa, with, however, some loss of quality in both. Objectives used for dark-field work must be of relatively low numeric aperture (usually less than 0.9); hence, when the ordinary oil-immersion objective is used, its aperture must be reduced by placing in it a "funnel stop" obtainable from the maker of the objective. Oil-immersion objectives of low N. A., especially designed for this work, but also useful for bright-field work, have recently been placed on the market. Leitz now makes a dark-field condenser which permits the use of numeric apertures up to 1.5.

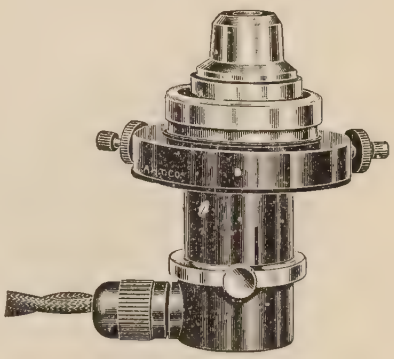


FIG. 7.—Dark-field condenser with lamp attached.

The chief use of dark-field illumination in clinical work is for demonstration of *Treponema pallidum* in fresh material (Fig. 277), and intestinal flagellates in feces.

Method of Using Dark-field Condenser.—A small drop of the fluid to be examined is placed on a clean slide[1] of the correct thickness

[1] As a desirable substitute for the usual glass slide many makers now supply a glass chamber in a metal holder. This is somewhat similar to the hemacytometer, but is of a depth suitable for dark-field work. The cover-glass is held in place by means of spring clips. This device insures correct thickness both for the slide and for the layer of fluid under examination. For critical work with the highest type of condenser the chamber is made of fused quartz.

and covered with a clean cover-glass. The thickness of the slide is important owing to the need of accurately focusing the condenser, and the proper thickness to be used with a particular condenser is generally engraved upon its mounting. This is usually between 1 and 1.55 mm. The layer of fluid must be thin. The slide and cover must be free from scratches; air-bubbles must be avoided, and also any excess of objects (blood-corpuscles, pus cells, and so forth) other than those which are sought, since all of these tend to brighten the background and thus reduce contrast.

The source of light may be direct sunlight, or, preferably, a strong artificial light with bull's-eye or water-bottle condenser to deliver parallel rays to the mirror. When the arc light is used, it is desirable to interpose a piece of lightly oiled ground-glass between the light and

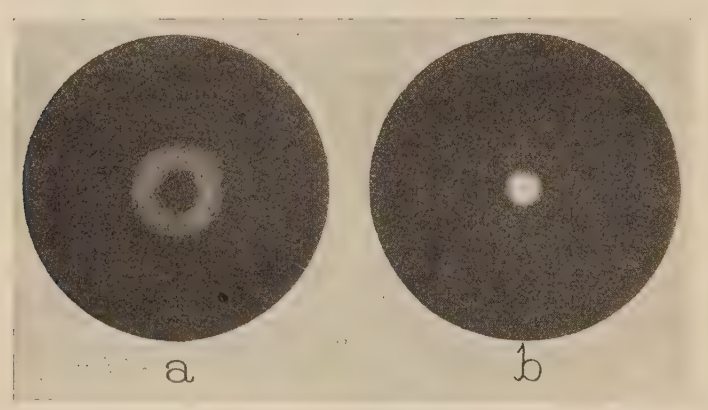


FIG. 8.—Dark-field illumination: a, Circle of light seen with a low-power objective when the condenser is above or below the correct focus. b, The bright spot of light seen with a low-power objective when the condenser is correctly focused.

the mirror, but this is not to be inserted until after the condenser has been focused.

The regular substage condenser is removed, the dark-field condenser is inserted in its place, and accurately centered in the optic axis. To facilitate centering, a series of concentric circles is generally ruled on the top of the condenser. These circles are brought to the center of the field of a low-power objective by means of centering screws provided for the purpose. A drop of immersion oil is then placed on the apex of the condenser, the slide is placed in position on the stage, and the condenser is raised until the oil is in contact with the under surface of the slide. The low-power objective is now focused on the slide. If the light be sufficiently intense and the mirror properly adjusted, a circle or a spot of light should be seen in the center of the field. A circle

indicates that the condenser is decidedly above or below its correct position (Fig. 8). The condenser is then focused by raising or lowering it until the circle becomes a spot of light and this spot becomes as small and as bright as it is possible to make it. This spot is also utilized for centering such condensers as are not provided with the concentric centering circles mentioned above. The low-power objective is finally replaced by the higher power with which the examination is to be made and this is brought to focus and used in the ordinary way.

For dark-field work the various adjustments must be much more exact than for bright field. The most frequent causes of failure to secure a satisfactory dark field with brilliantly lighted objects which appear to be self-luminous are:

1. Use of an objective of too high aperture. When the regular oil-immersion objective is used, its aperture must be reduced by means of the stop provided by the makers.

2. Failure accurately to focus and center the condenser. Very slight readjustments of condenser or mirror after the examination is begun may remedy matters, provided the slide is not too thick to permit accurate focusing.

3. Inclusion of air-bubbles in the preparation or in the oil above or below the slide. It is generally necessary to remove the oil and apply again.

4. Inclusion of too many microscopic objects in the field. This may be remedied by diluting the fluid to be examined, or by reducing the thickness of the preparation by means of slight pressure on the cover-glass.

The Condenser.—For the work of the clinical laboratory a substage condenser is a necessity. Its purpose is to condense the light upon the object to be examined. For critical work the light must be focused on the object by raising or lowering the condenser by means of the screw provided for the purpose. The image of the light source will then appear in the plane of the object. This is best seen by using a low-power objective and ocular. Should the image of the window-frame or other nearby object appear in the field and prove annoying, the condenser may be raised or lowered a little. It is often advised to remove the condenser for certain kinds of work, but this is not necessary and is seldom desirable in the clinical laboratory.

The condenser is constructed for parallel rays of light. With daylight, therefore, the plane mirror should be used, while for the

divergent rays of ordinary artificial light the concave mirror, which tends to bring the rays together, is best.

It is very important that the condenser be accurately centered in the optical axis of the instrument, and most high-grade instruments have centering screws by which it can be adjusted at any time. The simplest way to recognize whether the condenser is centered is to close the diaphragm beneath it to as small an opening as possible, then remove the eye-piece and look down the tube. If the diaphragm-opening does not appear in the center of the field, the condenser is out of center.

The use of the condenser is further discussed in the following sections.

Objectives and Eye-pieces.—Unfortunately, different makers use different systems of designating their lenses. The system used in this country is to designate objectives by their focal lengths in millimeters or by their "initial magnification," and eye-pieces by their magnifying power, indicated by an "×." Most foreign makers use this system for their high-grade lenses, but still cling to arbitrary letters or numbers for the others.

Objectives are of two classes—achromatic and apochromatic. Those in general use are of the achromatic type, and they fulfil all requirements for ordinary work. Apochromatic objects are more highly corrected for chromatic and spheric aberration, and represent the highest type of microscope lenses produced. They give crisp images with little or no trace of the color fringes which with achromatic objectives can readily be seen about the edges of black or colorless objects lying in a bright field, and are hence very desirable for photomicrography and research; but for routine laboratory work they do not offer advantages commensurate with their great cost. Within recent years, by use of fluorite in conjunction with special glasses, a third type of objective whose color corrections and price are midway between the achromats and apochromats, has been constructed. This is sometimes designated as a "semi-apochromat."

The simple eye-pieces which have long been used with achromatic objectives are known as Huygenian oculars. With apochromatic objectives it is necessary to use special "compensating eye-pieces" which are corrected to overcome certain defects in this type of objective. The same compensating eye-pieces may

be used with oil-immersion and high dry objectives of the achromatic series. A third type of ocular has recently been introduced under the trade names "hyperplane," "periplan," and so forth. They have a compensation midway between the compensating and Huygenian eye-pieces and may be used with either achromatic or apochromatic objectives. Their chief advantage is that they overcome to a very marked degree the curvature of field of any objective with which they may be used.

Objectives are "corrected" for use under certain fixed conditions, and *they will give the best results only when used under the conditions for which corrected.* The most **important corrections** are: (1) for tube length, (2) for thickness of cover-glass, and (3) for the medium between objective and cover-glass.

1. The tube length with which an objective is to be used is usually engraved upon it—in most cases it is 160 mm. The draw-tube of the microscope should be pulled out until the proper length (measured with ocular and objective removed) is obtained. The length is indicated by the graduations upon the side of the draw-tube, but in some cases this scale is made for use without a nose-piece. When a nose-piece is attached it adds about 15 mm. to the tube length.

2. The average No. 2 cover-glass is about the thickness for which most objectives are corrected—usually 0.17 or 0.18 mm. One can get about the right thickness by buying No. 2 covers and discarding the thick ones, or by buying No. 1 covers and discarding the thinner ones. Slight differences in cover-glass thickness can be compensated by increasing the length of tube when the cover is too thin, and decreasing it when the cover is too thick. This should be done with a spiral motion while supporting the body-tube with the other hand. The amount of correction necessary will depend upon the focal length and numeric aperture of the objective. With a 4-mm. objective of 0.85 numeric aperture a difference of 0.03 mm. in cover-glass thickness requires a change of 30 mm. in the tube length. Many high-grade objectives are supplied with a "correction collar," which accomplishes the same end. While for critical work, especially with apochromatics, cover-glass thickness is very important, one pays little attention to it in the clinical laboratory. A high-power dry lens always requires a cover, but its exact thickness is unimportant in routine work.

Very low-power and oil-immersion objectives may be used without any cover-glass.

3. The correction for the medium between objective and cover-glass is very important. This medium may be either air or some fluid, and the objective is hence either a "dry" or an "immersion" objective. The immersion fluid generally used is an especially prepared cedar oil, which gives great optical advantages because its index of refraction is the same as that of crown glass. It is obvious that only objectives with very short working distance, as the 2 mm., can be used with an immersion fluid.

To use an oil-immersion objective a suitable field for study should first be found with the low power. A drop of immersion oil is placed on the slide and the objective lowered until it is in contact with the oil and almost touches the slide. This is observed with the eye on a level with the stage. Then, with the eye looking into the microscope, the objective is very slowly raised until the objects on the slide are in focus. In order to avoid air-bubbles the oil must be placed on the slide carefully and without stirring it. Bubbles are a frequent source of trouble, and should always be looked for when an immersion objective does poor work. They are readily seen by removing the eye-piece and looking down the tube. If they are present, the oil must be removed and a new drop applied. Immediately after use both objectives and slide should be wiped clean with lens paper or a soft linen handkerchief. In an emergency glycerin may be used instead of cedar oil, but, of course, with inferior results.

Curvature of field, through which it is impossible to focus both center and periphery sharply at the same time, is a very noticeable defect; but it is less serious than appears at first sight, particularly for visual work. It is easiiy compensated by frequent use of the fine focusing adjustment. Complete flatness of field cannot be attained without sacrifice of other and more desirable properties. Some of the finest objectives made, notably the apochromatics, show decided curvature.

The **working distance** of an objective should not be confused with its focal distance. The former term refers to the distance between the front lens of the objective, when it is in focus, and the cover-glass. It is always less than the focal distance, since the "focal point" lies somewhere within the objective; and it varies

considerably with different makes. Long working distance is a very desirable feature. Some oil-immersion objectives have such short working distance that only very thin cover-glasses can be used.

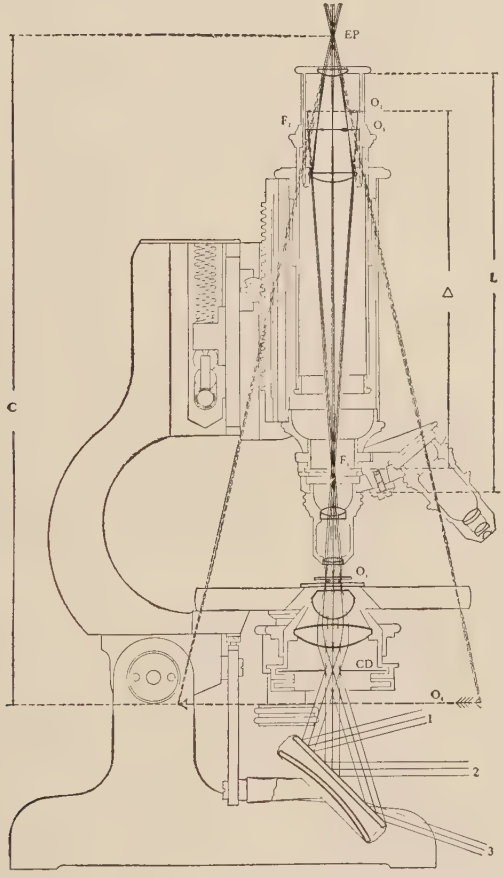


FIG. 9.—Diagram showing path of light rays: F_1, Upper focal plane of objective; F_2, lower focal plane of eye-piece; △, optical tube length = distance between F_1 and F_2; O_1, object; O_2, real image in F_2, transposed by the collective lens, to O_3, real image in eye-piece diaphragm; O_4, virtual image formed at the projection distance C, 250 mm. from EP, eyepoint; CD, condenser diaphragm; L, mechanical tube length (160 mm.); 1, 2, 3, three pencils of parallel light coming from different points of a distant illuminant, for instance, a white cloud, which illuminate three different points of the object.

The **formation of the microscopic image** demands brief consideration (Fig. 9). The rays of light which are reflected upward from the mirror and which pass through the object are brought to a focus in a magnified, inverted real image. This can be focused to

appear at different levels, but when the microscope is used in the ordinary way it is formed at about the level of the diaphragm in the ocular. It can be seen by removing the ocular, placing a piece of ground glass on the top of the tube, and focusing upon it. When viewing this image a roll of paper or a cylindric mailing tube should be used to exclude extraneous light. This image, in turn, is magnified by the eye-lens of the ocular, producing a second real image, which is again inverted, and, therefore, shows the object right side up. This can be seen upon a ground glass held a few inches above the ocular, provided strong artificial light be used and the room darkened. The eye, when it looks into the microscope, sees, not this real image, but rather an inverted *virtual image* which appears about 250 mm. (10 inches) in front of the eye.

Numeric Aperture.—This expression, usually written N. A., indicates the amount of light which enters an objective from a point in the microscopic field. In optical language N. A. is the sine of one-half the angle of aperture multiplied by the index of refraction of the medium between the cover and the front lens. Numeric aperture is extremely important, because upon it depends *resolving power*, which is the most important property of an objective.[1]

Resolving power is the ability to separate minute details of structure. For example, the dark portions of a good half-tone picture appear gray or black to the unaided eye, but a lens easily resolves this apparently uniform surface into a series of separate dots. Resolving power does not depend upon magnification. The fine lines and dots upon certain diatoms may be brought out clearly and crisply (that is, they are resolved) by an objective of high numeric aperture, whereas with an objective of lower numeric aperture, but greater magnifying power, the same diatom may appear to have a smooth surface, with no markings at all, no matter how greatly it is magnified. Knowing the N. A., it is possible to calculate how closely lines and dots may lie and still be resolved by a given objective. To state the numeric aperture, therefore, is to tell what the objective can accomplish, provided, of course, that spheric and chromatic aberrations are satisfactorily corrected. An objective's N. A. is usually engraved upon the mounting.

[1] Resolving power really depends on two factors, the N. A. and the wave length of light, but the latter can be ignored in practice.

It is an important fact, and one almost universally overlooked by practical microscopists, that the proportion of the numeric aperture of an objective which is *utilized* depends upon the aperture of the cone of light delivered by the condenser. In practice, the numeric aperture of an objective is reduced nearly to that of the condenser (which is indicated by lower case letters, n. a.).[1] The condenser should, therefore, have a numeric aperture at least equal to that of the objective with which it is to be used. Lowering the condenser below its focal distance and closing the diaphragm beneath it have the effect of reducing its working aperture. A condenser, whatever its numeric aperture, cannot deliver through the air a cone of light of greater N. A. than 1. From these considerations it follows that the proper adjustment of the substage condenser is a matter of great importance when using objectives of high N. A., and that, to gain the full benefit of the resolving power of such objectives, the condenser must be focused on the object under examination, it must be oiled to the under surface of the slide in the same way as the immersion objective is oiled to the coverglass, and the substage diaphragm must be wide open. The last condition introduces a difficulty in that colorless structures will appear "fogged" in a glare of light, making a satisfactory image impossible when the diaphragm is more than three-quarters open (Fig. 6). Wright suggests that the size of the light source be so regulated by a diaphragm that its image, thrown on the slide by the condenser, coincides with the real field of the objective, and maintains that in this way it is possible to reduce the glare of light and to dispel the fog without closing the diaphragm.

One can easily determine how much of the aperture of an objective is in use by removing the eye-piece, looking down the tube, and observing what proportion of the back lens of the objectives is illuminated. The relation of the illuminated central portion to the unilluminated peripheral zone indicates the proportion of the numeric aperture in use. The effect of raising and lowering the condenser and of oiling it to the slide can thus be easily seen.

Another property of an objective which depends largely upon N. A. is **depth of focus,** the ability to render details in different

[1] The N. A. of the objective is not reduced wholly to that of the condenser, because, owing to diffraction phenomena, a part of the unilluminated portion of the back lens is utilized.

planes clearly at the same time. The higher the N. A. and the greater the magnification, the less the depth of focus. Any two objectives of the same focal length and same N. A. will have exactly the same depth of focus. Depth of focus can be increased by closing down the diaphragm, and thus reducing the N. A. Great depth is desirable for certain low-power work, but for high powers it does not offer advantages to balance the loss of N. A. by which it is attained. In some cases, indeed, it is a real disadvantage.

Magnification.—The degree of magnification should always be expressed in *diameters*, not *times*, which is a misleading term. The former refers to increase of *diameter;* the latter, to increase of *area*. The comparatively low magnification of 100 diameters is the same as the apparently enormous magnification of 10,000 times.

According to the system of rating magnification in use in this country, the magnifying power of an objective is ascertained by dividing the *optical tube length* (Δ in Fig. 9) by the focal length of the objective. The optical tube length is usually somewhere near 165 mm., but it varies with the different objectives; and the makers' catalogs must be consulted for an accurate statement of magnifying power. Some makers follow the commendable plan of engraving both the focal length of the objective and its initial magnification upon its barrel.

This system of rating magnification measures the enlarged image at the level of the diaphragm in the ocular, and this image is, in turn, magnified by the ocular, so that when an objective and ocular are used together the total magnification is the product of the two. In the case, for example, of the 1.9 mm. oil-immersion objective, whose initial magnification is 95 diameters, the total magnification with the 5× ocular is 475 diameters.

It is easy to find the magnifying power of any combination of objective and ocular by actual trial. Place the counting slide of the hemacytometer upon the microscope and focus the ruled lines. Now adjust a sheet of paper upon the table close to the microscope in such a position that when the left eye is in its proper place at the ocular the paper will lie in front of the right eye at the normal visual distance, that is, 250 mm. (10 inches). The paper may be supported upon a book, if necessary. If both eyes are kept open, the ruled lines will appear to be projected on the paper. With a pencil mark on the paper the apparent location of the lines which bound the small squares used in counting

red blood-corpuscles and measure the distance between the marks. Divide this distance by 0.05 mm., which is the actual distance between the lines on the slide. The quotient gives the magnification. If, to take an example, the lines in the image on the paper are 5 mm. apart, the magnification is 100 diameters. The figures obtained in this way will vary somewhat as one is near- or far-sighted, unless the defect of vision is corrected with glasses.

In practice, magnification can be increased in one of three ways:

1. *Drawing Out the Tube.*—Since the increased tube length interferes with spheric correction, it should be used only with the knowledge that an imperfect image will result.

2. *Using a Higher Power Objective.*—As a rule, this is the best way, because resolving power is also increased; but it is often undesirable because of the shorter working distance, and because the higher objective often gives greater magnification than is desired, or cuts down the size of the real field to too great an extent.

3. *Using a Higher Power Eye-piece.*—This is the simplest method. It has, however, certain limitations. When too high an eye-piece is used, there results a hazy image in which no structural detail is seen clearly. This is called "empty magnification," and depends upon the fact that the objective has not sufficient resolving power to support the high magnification. It has been aptly compared to the enlargement, by stretching in all directions, of a picture drawn upon a sheet of rubber. No new detail is added no matter how great the enlargment. The extent to which magnification can be satisfactorily increased by eye-piecing depends wholly upon the resolving power of the objective, and consequently upon the N. A. The greatest total or combined magnification which will give an *absolutely* crisp picture is found by multiplying the N. A. of an objective by 400. The greatest magnification which can be used at all satisfactorily is 1,000 times the N. A. For example: The ordinary 1.9-mm. objective has a N. A. of 1.30; the greatest magnification which will give an absolutely sharp picture is 520 diameters, which is obtained approximately by using a 5.5 × eye-piece. Higher eye-pieces can be used, up to a total magnification of 1,300 diameters (12.5× eye-piece), beyond which the image becomes wholly unsatisfactory.

The Microscope in Use.—Optically, it is a matter of indifference whether the instrument be used in the vertical position or in-

clined. Examination of fluids requires the horizontal stage, and since much of the work of the clinical laboratory is of this nature it is well to accustom one's self to the use of the vertical microscope. While working one should sit as nearly upright as is possible compatible with comfort, and the height of the seat should be adjusted with this in view.

It is always best to "focus up," which saves annoyance and probable damage to slides and objectives. This is accomplished by bringing the objective nearer the slide than the proper focus, and then, with the eye at the eye-piece, turning the tube up until the object is clearly seen. *The fine adjustment should be used only to get an exact focus with the higher power objectives after the instrument is in approximate focus.* It should not be turned more than one revolution.

There will be less fatigue to the eyes if both are kept open while using the microscope, and if no effort is made to see objects which are out of distinct focus. Fine focusing should be done with the fine adjustment, not with the eye. An experienced microscopist keeps his fingers almost constantly upon one or other of the focusing adjustments.

Although the ability to use the eyes interchangeably is sometimes very desirable, greater skill in recognizing objects will be acquired if the same eye be always used. The left eye is the more convenient, because the right eye is thus left free to observe the drawing one may wish to do with the right hand. After a little practice one can cause the microscopic image to appear as if projected upon a sheet of paper placed close to the microscope under the free eye. This gives the effect of a camera lucida, and it becomes very easy to trace outlines. When one is accustomed to spectacles they should not be removed.

It is very desirable that one train himself to work with the low-power objective as much as possible, reserving the higher powers for detailed study of the objects which the low power has found. This makes both for speed and for accuracy. A search for tube-casts, for example, with the 4-mm. objective is both time consuming and liable to failure. Even such minute structures as nucleated red corpuscles in a stained blood-film are more quickly found with an 8-mm. or even a 16-mm. objective combined with a high ocular than with the oil-immersion lens. It is difficult for one who has not

measured it to realize how small is the "real field," that is, the actual area of the slide which is seen through the microscope (Fig. 10).

To be seen most clearly, an objective should be brought to the center of the field. *Acuity of vision will be greatly enhanced and fatigue lessened if all light except that which enters through the microscope be excluded from both eyes.* Strong light should not be allowed to fall directly upon the surface of the slide, as this clouds the image, especially with low powers. To this end various eye-shades have been devised, and some workers go so far as to work inside a small tent constructed of strips of wood covered with black cloth, the source of illumination being placed outside the tent.

A useful **pointer** can be made by placing a straight piece of a hair across the opening of the diaphragm of the eye-piece, cementing one end with a tiny drop of balsam, and cutting the hair in two in the middle with small scissors. When the eye-piece is in place, the hair appears as a black line extending from the periphery to the center of the microscopic field. If the pointer does not appear sharply defined it is out of focus, and the diaphragm must be raised or lowered a little within the ocular.

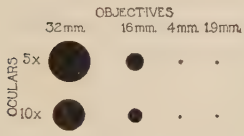


FIG. 10.—Showing the size of the "real fields" (actual areas seen through the microscope) with various objectives and oculars and tube length of 160 mm. The size differs slightly with different makes.

One often wishes to mark a particular field upon a permanent preparation so as to refer to it again. The vernier of the mechanical stage cannot be relied upon, because it is impossible to replace the stage in exactly the same position after it has been removed, and because its position is frequently changed by the slight knocks which it receives. There are on the market several "object markers" by which a desired field can be marked with ink, or by a circle scratched on the cover-glass by a minute diamond, while the slide is in place on the microscope. The circle is easily located with a low power. In the absence of these one can, while using the low power, place minute spots with a fine pen at the edge of the field on opposite sides.

A good marking material is a cement which Todd has long used for making cells, ringing cover-glasses, and so forth. To a few ounces of white shellac in wood alcohol add an equal volume of gasoline, shake thoroughly, and let stand for twenty-four hours, or until well separated into two layers. Pipet off the clear lower portion, add 5 to 10

drops of castor oil to each ounce, and color with any anilin dye dissolved in absolute alcohol. When too thick, thin with alcohol. This makes a beautiful, transparent, easy-flowing cement which does not crack and which is not readily attacked by xylol. Glycerin mounts ringed with it in 1895 are still in perfect condition.

Many good workers advise against the use of spring clips to hold the slide against the stage of the microscope. Manipulation of the slide with the fingers alone certainly gives good training in delicacy of touch, and is desirable when examining infectious material which might contaminate the clips, or when one must detect slight pressure of the objective upon the cover-glass, as in studying a hanging-drop preparation. For the majority of examinations, however, it is more satisfactory to use a clip at one end of the slide, with just sufficient pressure to hold the slide without interfering with its freedom of movement.

Occasionally when one wishes a very low-power objective for some special work it may be desirable to unscrew the front lens of the 16-mm. objective and use the back lens only. This procedure is not recommended for critical work, and it should not be tried with high-power objectives, *which must never be taken apart.*

To attach an objective it should be supported in position against the nose-piece by means of the index-finger and middle finger, which grasp it as one would a cigar. It is then screwed into place with the fingers of the other hand.

Care of the Microscope.—The microscope is a delicate instrument and should be handled accordingly. Even slight disturbance of its adjustments may cause serious trouble. It is so heavy that one is apt to forget that parts of it are fragile. It seems unnecessary to say that when there is unusual resistance to any manipulation, force should never be used to overcome it until its cause has first been sought; and yet it is no uncommon thing to see students, and even graduates, push a high-power objective against a microscopic preparation with such force as to break not only the cover-glass, but even a heavy slide.

To bend the instrument at the joint the force should be applied to the pillar and never to the tube or the stage.

The microscope should be kept scrupulously clean, and dust must not be allowed to settle upon it. When not in use the instrument should be kept in its case or under a cover. An expensive

glass bell-jar is not needed, and, in fact, is undesirable, except for display. It is heavy and awkward to handle, and when lifted is almost certain (unless great care is exercised) to strike the microscope. It is particularly liable to strike the mechanical stage and disturb its adjustment. The simplest, cheapest, lightest, and probably the best cover for the microscope is a truncated cone or pyramid of pasteboard, covered with creton or similar material. This is easily made at home. In the absence of a special cover a square or lintless cloth may be draped over the microscope.

Lens surfaces which have been exposed to dust only should be cleaned with a camel's-hair brush. A small brush and a booklet of lens-paper should always be at hand in the microscope case. Those surfaces which are exposed to finger-marks should be cleaned with lens-paper, or a soft linen handkerchief, moistened with water if necessary. The rubbing should be done very gently and with a circular motion. Particles of dirt which are seen in the field are upon the slide, the eye-piece, or the condenser. Their location can be determined by moving the slide, rotating the eye-piece, and lowering the condenser. Dirt on the objective cannot be seen as such; it causes a diffuse cloudiness. When the image is hazy, the objective probably needs cleaning; or in case of an oil-immersion lens, there may be bubbles in the oil.

Oil and balsam which have dried upon the lenses—an insult from which even dry objectives are not immune—may be removed with alcohol or xylol; but these solvents must be used sparingly and carefully, as there is danger of softening the cement between the components of the lens. Some manufacturers now claim to use a cememt which resists xylol. Care must be taken not to get any alcohol upon the brass parts, as it will remove the lacquer. Balsam and dried oil are best removed from the brass parts with xylol.

When the vulcanite stage becomes brown and discolored the black color can be restored by rubbing well with petrolatum.

Measurement of Microscopic Objects.—The importance of size in identification of microscopic structures cannot be too strongly emphasized. Even very rough measurements will often prevent humiliating blunders. The principal microscopic objects which are measured clinically are animal parasites and their ova and abnormal blood-corpuscles. The metric system is used almost exclusively.

For very small objects, 0.001 mm. has been adopted as the unit of measurement, under the name *micron*. It is represented by the Greek letter μ. For larger objects, where exact measurement is not essential, the diameter of a red blood-corpuscle (7 to 8 μ) is sometimes taken as a unit. Of the several methods of measurement, the most convenient and accurate is the use of a micrometer eye-piece. In its simplest form this is similar to an ordinary eye-piece, but it has within it a glass disk upon which is ruled a graduated scale. When this eye-piece is placed in the tube of the microscope, the ruled lines appear in the microscopic field, and the size of an object is readily determined in *terms of the divisions of this scale.* The value of these divisions in millimeters manifestly varies with different magnifications. Their value must, therefore, be determined separately for each objective. This is accomplished through use of a stage micrometer—a glass slide with carefully ruled scale divided into subdivisions, usually hundredths of a millimeter. The stage micrometer is placed upon the stage of the microscope and brought into focus. The tube of the microscope is then pushed in or pulled out until two lines of the one scale exactly coincide with two lines of the other. From the number of divisions of the eye-piece scale which then correspond to each division of the stage micrometer the value of the former in micra or in fractions of a millimeter is easily calculated. *This value, of course, holds good only for the objective and the tube length with which it was found.* The counting slide of the hemacytometer will answer in place of a stage micrometer, the lines which form the sides of the small squares used in counting red blood-corpuscles being 50 micra apart. When using the counting chamber with an oil-immersion lens a cover must be used, otherwise the oil will fill the ruled lines and cause them to disappear. Any eye-piece can be converted into a micrometer eye-piece by placing a micrometer disk—a small circular glass plate with ruled scale—ruled side down upon its diaphragm. If the lines upon this are at all hazy the disk has probably been inserted upside down, or else the diaphragm is out of its proper position. Usually it can be pushed up or down as required. The new "step" micrometer eye-piece is very satisfactory. The step-like arrangement of the scale (Fig. 11) makes it easy to read, and the divisions are such that they read in micra or easy multiplies of micra with little or no change from the regular tube length.

The following method of micrometry is less accurate, but is fairly satisfactory for comparatively coarse objects, such as the ova of parasites. A ruled scale corresponding to the magnified image of the hemacytometer ruling is drawn upon cardboard in the manner described for ascertaining magnifications (p. 32), except that the card is placed upon the table beside the microscope and not necessarily at a distance of 10 inches from the eye. This card may then be used as a micrometer, and should be inscribed with the value of its graduations, and the objective, ocular, and tube length with which it is to be used. In the example cited upon page 32 the lines on the card are 5 mm. apart, corresponding to an actual distance of 50 μ. To measure an object, the cardboard is placed in the position which it occupied when made (upon the table at the right of the microscope). The lines and the objects on the slide can then be seen together, and the space covered by any object indicates its size. The graduations made as above indicated are too coarse for most work, and they should be subdivided. If five subdivisions are made, each will have a value of 10 μ.

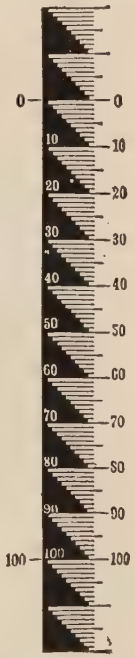


FIG. 11.—Scale of the step micrometer eye-piece.

Tuttle has suggested that in fecal and other examinations a little lycopodium powder be mixed with the material. The granules are of fairly uniform size—30 μ in diameter—and are easily recognized (Fig. 12). They furnish a useful standard with which the size of other structures can be compared. Care must be exercised not to use too much powder. The lycopodium is conveniently kept in a gelatin capsule, and a faint cloud can be dusted over the slide by gently scraping the edge of the lid upon the rim of the capsule.

Photomicrography.—Although high-grade photomicrography requires expensive apparatus and considerable skill in its use, fairly good pictures of microscopic structures can be made by any one with simple instruments.

Any camera with focusing screen or a Kodak with plate attachment may be used. It is best, but not necessary, to remove the photographic lens. The camera is placed with the lens (or lens opening, if the lens has been removed) looking into the eye-piece

of the microscope, which may be in either the vertical or the horizontal position. One can easily rig up a standard to which the camera can be attached in the proper position by means of a tripod screw. A light-tight connection can be made of a cylinder of paper or a cloth sleeve with drawstrings. The image will be thrown upon the ground-glass focusing screen, and is focused by means of the fine adjustment of the microscope. The degree of magnification is ascertained by placing the ruled slide of the blood-counting instrument upon the microscope and measuring the image on the screen. The desired magnification is obtained by changing objectives or eyepieces or lengthening the camera-draw.

Focusing is comparatively easy with low powers, but when using an oil-immersion objective it is a difficult problem unless the source

FIG. 12.—Egg of *Tænia saginata*. Lycopodium granules used as micrometer as described in the text. Since the granules average 30 μ in diameter, the egg is seen by comparison to be about 35 by 40 μ (photograph $\times$ 250).

of light be very brilliant. If one always uses the same length of camera and microscope tube, a good plan is as follows: Ascertain by trial with a strong light how far the fine adjustment screw must be turned from the correct eye focus to bring the image into sharp focus upon the ground-glass screen. At any future time one has only to focus accurately with the eye, bring the camera into position, and turn the fine adjustment the required distance to right or left. When the camera-draw is 10 inches little or no change in the focusing adjustment will be necessary.

The light should be as intense as possible in order to shorten exposure, but any light that is satisfactory for ordinary microscopic work will answer. The light must be carefully centered.

It is nearly always necessary to insert a colored filter between the light and the microscope. Pieces of colored window-glass are useful for this purpose, but much better filters can be purchased at trifling cost either as finished filters or in the form of gelatin sheets which can be cemented between glass plates with balsam. The filter should have a color complementary to that which it is desired to bring out strongly in the photograph: for blue structures, a yellow filter; for red structures, a green filter. For the average stained preparation, a picric-acid yellow or a yellowish green will be found satisfactory. For deeply stained objects whose strength it is desired to reduce, and for all objects within which it is desired to bring out as much of the internal detail as possible, a filter of the same color should be used.

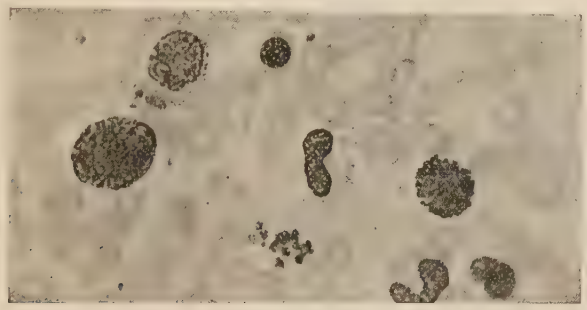


FIG. 13.—Leukemic blood (about × 650). Photograph taken with a Kodak, as described in the text.

Very fair pictures can be made on Kodak film, but orthochromatic plates (of which Cramer's "Iso" and Seed's "Ortho" are examples) give much better results. Panchromatic plates like the Wratten "M" are still better, but are more difficult to handle because more sensitive to red light. In order to avoid halation all plates should if possible be "backed." The length of exposure depends upon so many factors that it can be determined only by trial. It will probably vary from a few seconds to fifteen minutes. Plates are developed in the usual way. Either the tray or tank method may be used, but in order to secure good contrast it is often desirable to overdevelop somewhat. Metol-hydrochinon is an excellent developer, as it gives good contrast with full detail.

The photograph from which Figure 13 was made was taken with a Kodak and plate attachment on an "Iso" plate, the source of light being the electric lamp and condensing lens illustrated in

Figure 3. It was focused by the method described above. The screen was a picric-acid stained photographic plate. Exposure, three and a half minutes. The picture loses considerable detail in reproduction.

Choice of a Microscope.—It is poor economy to buy a cheap instrument.

For work in a clinical laboratory the microscope should preferably be of the handle-arm type, and should have a large stage. It should be provided with a substage condenser (preferably of 1.40 n. a.), three or more objectives on a revolving nosepiece, and two or more eye-pieces. After one has learned to use them, the new single objective binocular microscopes are extremely satisfactory, giving an impression of stereoscopic vision, also enabling the worker to keep both eyes open with no feeling of strain. Recently new, lower-priced types of binocular microscopes (Fig. 14) were placed on the market by American manufacturers. The distance between the two eye-pieces is adjustable to allow for differences in pupillary distances of different individuals; the left eye-piece may also be focused by turning it slightly and thus adjusting it for any slight refractive differences of the two eyes. If

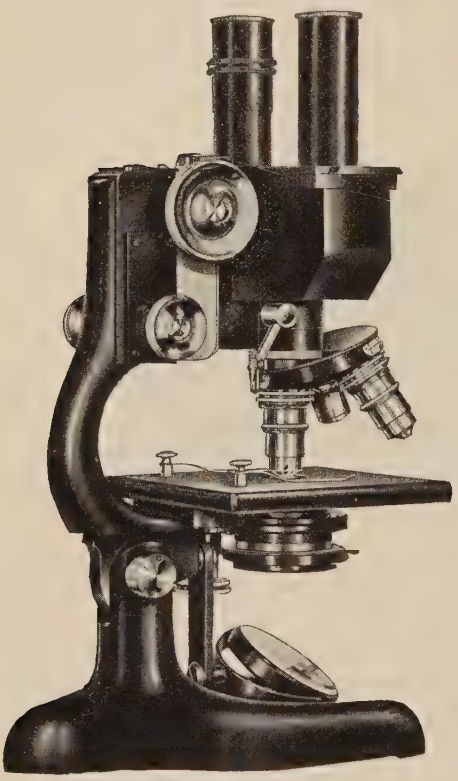


FIG. 14.—Binocular microscope.

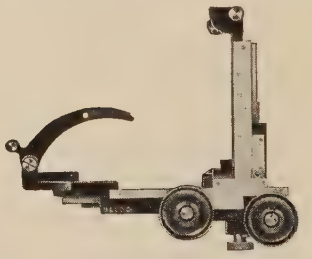


FIG. 15.—An attachable mechanical stage.

there is a bad refractive error it may be necessary to have special lenses ground for the eye-pieces to obtain stereoscopic effects. Microscopists generally prefer binocular to monocular instruments when they become familiar with their use.

The most generally useful objectives are: 16 mm., 4 mm., and 2 mm. oil immersion. The 4-mm. objective may be obtained with N. A. of 0.65 to 0.85. If it is to be used for blood-counting, the former is preferable, since its working distance is sufficient to take the thick cover of the blood-counting instrument. For coarse objects a 32-mm. objective is very desirable. The eye-pieces most frequently used are 5× and 10×. A very low power (2×) and a very high (15×) will sometimes be found useful. The micrometer eye-piece is almost a necessity. A mechanical stage, preferably of the attachable type, is almost indispensable for differential counting of leukocytes and certain other work of the clinical laboratory.

A first-class monocular microscope, of either American or foreign make, equipped as just described, will cost in the neighborhood of $125 to $135, exclusive of the mechanical stage; a binocular microscope will cost from $60 to $75 more.

Practical Exercises.—The following is a brief outline of certain exercises which have been found useful in teaching microscopy. The student must learn as early as possible what can be expected of his microscope with proper manipulation. When he sits down to work his first glance should tell him whether the instrument is giving its best results. If the microscopic picture falls short of the best, he must locate the difficulty and correct it before proceeding.

1. Clean the microscope and study its parts, familiarizing yourself with the names, purposes, and movements of each (Fig. 1).

2. Practice the manipulations necessary to locate particles of dust or dirt which appear in the microscopic field (p. 37).

3. Place the microscope before a window, focus upon a dusty slide, and adjust condenser and mirror so that the image of the window-frame or, better, of trees just outside the window appear in the microscopic field. Try the effect of raising and lowering the condenser, and of changing from plane to concave mirror, upon these images. Note that they cause an unevenly illuminated or mottled field when a little out of focus.

4. Insert a "pointer" in one of the oculars (p. 35).

5. Study illumination. Use a slide of some colorless structures, such as cholesterol crystals and two preparations of blood, one a dried film stained with eosin or any blood stain (p. 271) and mounted in balsam, the other an unstained wet preparation made as described for the malarial parasite (p. 314). Study only the areas in which the corpuscles are well separated.

(1) Place one of these on the microscope, bring to a focus, and practice the manipulations necessary to secure (p. 21)—

(*a*) Central illumination.

(*b*) Oblique illumination.

(*c*) Strong and subdued illumination.

The field in each case must be evenly lighted throughout without mottling. Continue until you can adjust any desired form of illumination quickly and surely, and can recognize each by a glance into the microscope.

(2) Using the three slides mentioned above, ascertain the best form of illumination to study (p. 22)—

(*a*) Outlines.

(*b*) Color.

(*c*) Surface contour. The unstained normal and crenated red corpuscles are excellent objects for study of surface contour (Figs. 93, 94).

(3) Try dark-field illumination by means of the substage disk (p. 22). Study the unstained blood-smear and draw a few corpuscles. Also examine the cholesterol crystals, a drop of diluted milk, and a bit of lens paper. Use the 16-mm. objective for this.

6. With central illumination, focus upon a slide and observe how much of the numeric aperture is in use (p. 31). Try the effect upon numeric aperture of—

(1) Opening and closing the diaphragm.

(2) Raising and lowering the condenser.

(3) Using the oil-immersion objective—

(*a*) Without oil.

(*b*) With oil between objective and cover-glass.

(*c*) With oil between slide and condenser.

7. Upon the same species of diatom compare two objectives of 3-mm. focus (therefore of same magnifying power), one of N. A. 1.4 and the other of N. A. 0.85. They will be adjusted by the instructor. Note the superior resolving power of the lens of high N. A. (p. 30).

8. Practice using the oil-immersion objective (p. 28) upon an un-

stained film preparation of blood or a slide strewn with diatoms. These are nearly colorless and hence difficult to see. If there is difficulty in finding the specimen, move the slide about while lowering the objective to a focus. Moving objects will catch the eye as the objective approaches the correct focus. If a cover-glass is used, its edge can be easily found, but it must be borne in mind that when the upper surface of the cover is in focus, objects beneath it are so far out of focus as to be invisible.

Produce some bubbles in the oil by stirring it about on the slide; observe their effect on the image of the blood-cells or diatoms, and learn to detect their presence (p. 28).

9. Image formation (p. 29). Mount a bit of paper printed with very small type, using oil or balsam to render it transparent. Focus upon this with a low-power objective. Remove the ocular and lay a piece of ground glass across the top of the tube. This forms a screen upon which an image can be focused by means of the coarse adjustment. If the ground glass be placed over only half of the top of the tube, half of the image can be seen on the screen, half in the air. Note whether it is right side up or reversed. Repeat this with the ocular in place, holding the ground glass some inches above the ocular. These exercises, especially the last, are best done in a darkened room with strong artificial illumination, but extraneous light can usually be sufficiently excluded by viewing the image through a pasteboard mailing cylinder.

10. Find by trial the magnification produced by your 16-mm. objective with the 4× or 6× ocular (p. 32). Compare your result with that listed by the maker of the microscope.

11. Micrometry.

(1) Evaluate the scale of your micrometer eye-piece with a high-power objective, and measure accurately 10 red blood-corpuscles and 10 leukocytes (p. 38).

(2) Prepare a cardboard micrometer and measure 10 lycopodium granules (p. 39).

12. Focus upon a stage micrometer or hemacytometer slide and measure the diameter of the real field of each of your objectives with each of the oculars (p. 35). Note the effect of increasing the tube length.

13. Study the following structures, chiefly with a view to best illumination. Examine separately the color, outline, and surface contour of each. Many of these are met as accidental contaminations in microscopic preparations and one must learn to recognize them. Make drawings of each.

Fluids are examined by placing a drop in the center of a clean slide and applying a cover-glass. The drop should be large enough to fill

the space between the slide and cover, but not large enough to float the cover about. Fibers or insoluble powder may be placed in a drop of water and covered.

(1) With 16-mm. and 4-mm. objectives examine the upper surface of a new cover-glass without cleaning. Usually it will show dirt and often crystals; if not, make finger-prints upon it and produce faint scratches by rubbing two covers together.

(2) Air-bubbles produced by shaking a little diluted mucilage.

(3) Fresh milk diluted with three or four volumes of water. Prepare three slides.

(*a*) Examine one untreated.

(*b*) Treat one with solution of sudan III. (For method see p. 172.) Note color assumed by the fat globules. This is one of the most useful tests for microscopic fat.

(*c*) Treat one with dilute acetic acid. Note clumping of globules similar to that of typhoid bacilli in the Widal test.

(4) A drop of diluted India-ink. Note the dancing motion of the smaller particles ("Brownian motion").

(5) Starch granules. Gently scrape the freshly cut surface of a potato with a knife, place a drop of the cloudy fluid upon a slide with a drop of water, and apply a cover-glass. Make two preparations.

(*a*) Examine one untreated. Note the variously sized starch granules, oval, colorless, concentrically striated. Make sure that you find the best form of illumination to bring out the striations clearly. The starch granules themselves are easy to see because of their broad dark outlines. This means that they are "highly refractive"—a term much used in describing microscopic structures—or, more correctly, that their index of refraction differs greatly from that of the medium in which they are mounted.

(*b*) Treat one with dilute Lugol's or Gram's iodin solution. Note the change in color of the granules. This is the standard test for starch.

(6) Yeast which has been growing in a dextrose solution. Make two preparations.

(*a*) Examine one unstained. Note "budding."

(*b*) Treat one with iodin solution. Compare color of yeast with that taken by starch.

(7) Mold from moldy food. Note hyphæ and spores. Try the effect of iodin.

(8) Various fibers and other structures mounted in a drop of water:

(*a*) Cotton.
(*b*) Wool.
(*c*) Linen.
(*d*) Silk.
(*e*) Feather tip.
(*f*) Some dust from a carpeted room. Colored fibers from the carpet are frequently found in urine.
(*g*) A hair.
(*h*) Pollen from as many species of flowers as possible.

(9) A drop of decomposing urine. Note bacteria of various kinds, some motile, some non-motile. Make an effort to distinguish true motility from that due to currents in the fluid and to "Brownian motion."

(10) Some of the scum from the bottom of a stagnant pool. Note the abundance of microscopic life. Look especially for diatoms, amebæ, and ciliated organisms.

(11) Test your proficiency in using the microscope by trying to resolve diatoms. For the 4-mm. objective use *Pleurosigma angulatum*. The dots should be clearly seen. For the oil-immersion lens use *Surirella gemma*. The fine lines between the ribs should be seen as rows of dots. As a most critical test, both of the oil-immersion lens and of your skill in manipulation, use *Amphipleura pellucida*. Select a diatom of large size. Use oblique illumination and endeavor to bring out the cross striations. Try the same with central light, although you are not likely to succeed. These striations consist of rows of extremely minute dots, which can be seen only under the most favorable conditions such as are rarely attained in clinical work.

A NOTE ON THE MAGNIFICATIONS USED FOR THE ILLUSTRATIONS

The appearance of microscopic structures can generally be well shown by photomicrographs or good drawings, but as to their size, which is equally important for their identification, the picture itself gives no hint unless some object which is familiar to us has

been included. Circular pictures, indeed, are often actually misleading. They generally represent only a small area from the center of the microscopic field of view; but to many persons they appear to represent the entire field, and objects which occupy a large portion of the picture are accordingly visualized as occupying a correspondingly large part of the microscopic field and therefore as being much larger than they really are.

In order to derive the greatest possible benefit from study of the illustrations of microscopic structures, it is necessary that the student train himself to interpret the size of the pictured objects in terms of the magnifications afforded by his own microscope. To this end, the magnifications at which these objects are depicted are indicated beneath the illustrations. For example, Figure 8 of Plate XI shows a tapeworm egg photographed at 250 diameters, and represents it about as it appeared with the 4-mm. objective and 6× ocular, and about twice as large as it appeared with a 16-mm. objective and 12.5-mm. ocular. The actual size of the object is found by measuring its picture and dividing by the stated magnification. In this case the pictured egg is about 9 mm. long, and the egg itself was approximately 36 μ long. In order to make up for unavoidable loss of detail in reproduction, the illustrations have in most cases been made at somewhat higher magnifications than are generally used for study of the objects in question. In many cases the same object is shown at both high and low magnifications.

The magnifications afforded by the different combinations of one's objectives and oculars can easily be found by the method given on page 32, or by consulting the maker's catalogue.

CHAPTER I

THE SPUTUM

Preliminary Considerations.—Before beginning the study of the sputum the student will do well to familiarize himself with the structures which may be present in the normal mouth, and which frequently appear in the sputum as contaminations. Nasal mucus and material obtained by scraping the tongue and about the teeth should be studied as described for unstained sputum. A drop of Lugol's solution should then be placed at the edge of the cover, and, as it runs under, the effect upon different structures noted. Another portion should be spread upon slides or covers and stained by some simple stain and by Gram's method. The structures likely to be encountered are epithelial cells of columnar and squamous types; leukocytes, chiefly mononuclear, the so-called salivary corpuscles; food-particles; *Leptotrichia buccalis;* great numbers of saprophytic bacteria; and frequently "spirochetes" and endamebæ. These structures are described later. The so-called "normal morning sputum" is described on page 80.

When **collecting the sample** for examination the morning sputum, or the whole amount for twenty-four hours, should be saved. In beginning tuberculosis tubercle bacilli can often be found in that first coughed up in the morning, when they cannot be detected at any other time of day. Sometimes, in these early cases, there are only a few mucopurulent flakes which contain the bacilli, or only a small purulent mass every few days, and these may easily be overlooked by the patient.

Patients should be instructed to rinse the mouth well, in order to avoid contamination with food-particles which may prove confusing in the examination, and to make sure that the sputum comes from the lungs or bronchi and not from the nose and nasopharynx. Many persons find it difficult to distinguish between the two sources. It is desirable that the material be raised with a distinct expulsive cough, but this is not always possible. In some cases of chronic

tuberculosis there may be no cough at all, the small masses of sputum rising by action of bronchial and tracheal cilia, and the patient becoming conscious of them only when they reach the larynx, and often swallowing them without realizing their significance. Material from the upper air-passages can usually be identified by the large proportion of mucus and the character of the epithelial cells.

The sputum of infants and young children is usually swallowed and therefore cannot be collected. In such cases examination of the feces for tubercle bacilli will sometimes establish a diagnosis of tuberculosis.

As a receptacle for the sputum a clean, wide-mouthed bottle with tightly fitting cork may be used. The patient must be particularly cautioned against smearing any of it upon the outside of the bottle. This is probably the chief source of danger to those who examine sputum. Disinfectants should not be added. Although some of them (phenol, for example) do not interfere with detection of tubercle bacilli, they generally so alter the character of the sputum as to render it unfit for other examinations.

The following outline is suggested for the **routine examination:**

1. Spread the material in a thin layer in a large Petri dish or between two plates of glass. The use of glass plates is messy, but is to be recommended for careful work. The top plate should be much smaller than the lower one, or have some sort of handle.

2. Examine all parts carefully with the naked eye or with a hand lens. This is best done over a black background which is placed some distance below. The ordinary paper picnic plate, one-half of which is painted black, makes a convenient tray, but the black background is too close for greatest efficiency. The portions most suitable for further examination may thus be easily selected. *This macroscopic examination should never be omitted.*

3. Transfer various portions, including all suspicious particles, to clean slides, cover, and examine unstained with the microscope (p. 54).

4. Slip the covers from some or all of the above unstained preparations, leaving a thin smear on both slide and cover.

5. Dry and fix the smears and stain one or more by each of the following methods:

(*a*) For tubercle bacilli (p. 64).

(*b*) Gram's method (p. 657).

6. When indicated, make special examinations for—
   (*a*) Capsules of bacteria (p. 73).
   (*b*) Eosinophilic cells (p. 78).
   (*c*) Much's granules (p. 70).
   (*d*) Spirochetes (p. 76).
   (*e*) Presence of albumin (p. 80).

After the examination the sputum must be destroyed by heat or chemicals, and everything which has come in contact with it must be sterilized. The utmost care must be taken not to allow any of it to dry and become disseminated through the air. If flies are about, it must be kept covered. It is a good plan to conduct the examination upon a large newspaper, which can then be burned. Contamination of the work table is thus avoided. If this is not feasible, the table should be washed off with 10 per cent. lysol or other disinfectant solution, and allowed to dry slowly, as soon as the sputum work is finished.

Examination of the sputum is most conveniently considered under four heads: I. Physical examination. II. Microscopic examination. III. Chemical examination. IV. Characteristics of the sputum in various diseases.

I. PHYSICAL EXAMINATION

1. Quantity.—The quantity expectorated in twenty-four hours varies greatly. It may be so slight as to be overlooked entirely in beginning tuberculosis. It is usually small in acute bronchitis and lobar pneumonia. It may be very large—sometimes as much as 1,000 c.c.—in advanced tuberculosis with large cavities, edema of the lung, bronchiectasis, and following rupture of an abscess or empyema. It is desirable to obtain a general idea of the quantity, but accurate measurement is unnecessary.

2. Color.—Since the sputum ordinarily consists of varying proportions of mucus and pus, it may vary from a colorless, translucent mucus to an opaque, whitish or yellow, purulent mass. A yellowish green is frequently seen in advanced phthisis and chronic bronchitis. In jaundice, in caseous pneumonia, and in slowly resolving lobar pneumonia it may assume a bright green color, due to bile or altered blood-pigment.

A red or reddish-brown color usually indicates the presence of blood or of a pigment derived from it. Bright red blood, most

commonly in streaks, is strongly suggestive of phthisis. It may be noted early in the disease and generally denotes an extension of the tuberculous process. One must, however, be on his guard against blood-streaked mucus or mucopus originating in nasopharyngeal catarrh. Tuberculous patients not infrequently mistake this for true sputum and become much worried because of it. Blood-stained sputum is also sometimes seen in bronchiectasis. A rusty red sputum is the rule in croupous pneumonia, and was at one time considered pathognomonic of the disease. Exactly similar material may be raised in pulmonary infarction. "Prune-juice" sputum is said to be characteristic of "drunkard's pneumonia." It at least indicates a dangerous type of the disease, as it is apparently referable to coincident edema of the lung. A brown color, due to altered blood-pigment, follows hemorrhages from the lungs, and is present, to greater or less degree, in chronic passive congestion of the lungs, which is most frequently due to a heart lesion.

Gray or black sputum is observed among those who work much in coal-dust, and is occasionally seen in smokers who are accustomed to "inhale."

3. Consistence.—According to their consistence sputa are usually classified as serous, mucoid, purulent, seropurulent, or mucopurulent, which names explain themselves. As a rule, the more mucus and the less pus and serum a sputum contains, the more tenacious it is.

The rusty sputum of croupous pneumonia is extremely tenacious, so that the vessel in which it is contained may be inverted without spilling it. The same is true of the almost purely mucoid sputum ("sputum crudum") of beginning acute bronchitis, and of that which follows an attack of asthma. A purely serous sputum, usually slightly blood tinged, is fairly characteristic of edema of the lungs.

Formerly much attention was paid to the so-called "nummular sputum." This consists of definite mucopurulent masses which flatten out into coin-like disks and sink in water. It is fairly characteristic of tuberculous and bronchiectatic cavities.

4. Layer Formation.—Some sputa show a striking tendency to separate into three sharply defined layers when a large volume is allowed to stand in a tall vessel. This is notably true in bronchiectasis, gangrene, and abscess of the lung.

5. Dittrich's Plugs.—While these bodies sometimes appear in the sputum, they are more frequently expectorated alone. They are yellowish or gray caseous masses, usually about the size of a pin-head, but sometimes reaching that of a bean. When crushed, they emit a foul odor. Microscopically, they consist of granular débris, fat globules, fatty acid crystals, and bacteria. They are formed in the bronchi, and are sometimes expectorated by healthy persons, but are more frequent in putrid bronchitis and bronchiectasis. The laity commonly regard them as evidence of tuberculosis. The similar caseous masses which are formed in the crypts of the tonsils are sometimes also included under this name.

FIG. 16.—Bronchial casts as seen when carefully spread out and viewed over a black background. Natural size. More frequently only broken pieces are found.

FIG. 17.—Unusually large and perfect bronchial cast. One-half natural size (Spencer).

6. Lung Stones.—At times during the course of chronic tuberculosis small calcified nodules of tuberculous tissue may be expectorated, and these constitute the great majority of the so-called "pneumoliths." Small foreign bodies, bits of clothing, and so forth, carried into the lung by gunshot and other injuries, may sometimes remain for years and finally ulcerate into a bronchus and be expectorated usually with hemorrhage.

7. Bronchial Casts.—These are branching, tree-like casts of the bronchi, frequently, but not always, composed of fibrin (Figs. 16 and 17). In color they are usually white or grayish, but may be reddish or brown from the presence of blood-pigment. Their size

varies with that of the bronchi in which they are formed. Casts 15 or more centimeters in length have been observed, but they are usually very much smaller. Ordinarily they are rolled into a ball or tangled mass, and can be recognized only by floating out in water—best over a black background—when their tree-like structure becomes evident. The naked-eye examination will usually suffice; occasionally a hand lens may be required.

Bronchial casts appear in the sputum in croupous pneumonia, in fibrinous bronchitis, and in diphtheria when the process extends into the bronchi. In diphtheria they are usually large. In fibrinous or chronic plastic bronchitis they are of medium size and usually of characteristic structure. Their demonstration is essential for the diagnosis of this disease. In some cases they may be found every day for considerable periods; in others, only occasionally. In almost every case of croupous pneumonia the casts are present in the sputum in variable numbers during the stage of hepatization and beginning resolution. Here they are usually small (0.5 to 1 cm. in length) and are often not branched.

II. MICROSCOPIC EXAMINATION

The portions most likely to contain structures of interest should be very carefully selected, as already described. *The few minutes spent in this preliminary examination will sometimes save hours of work later.* Opaque, white or yellow particles are almost frequently bits of food, but may be cheesy masses from the tonsils; particles, sometimes caseous, derived from tuberculous cavities and containing many tubercle bacilli and elastic fibers; Curschmann's spirals, or small fibrinous casts, coiled into little balls; or shreds of mucus with great numbers of entangled pus-corpuscles. The food particles most apt to cause confusion are bits of bread, which can be recognized by the blue color which they assume when touched with iodin solution.

Some structures are best identified without staining; others require that the sputum be stained.

A. Unstained Sputum

A careful study of the unstained sputum should be included in every routine examination. Unfortunately, it is almost universally neglected. It best reveals certain structures which are seen im-

perfectly or not at all in stained preparations. It gives a general idea of the other structures which are present, such as pus-corpuscles, eosinophils, epithelial cells, and blood, and thus suggests appropriate stains to be used later. It enables one to select more intelligently the portions to be examined for tubercle bacilli.

The particle selected for examination should be transferred to a clean slide, covered with a clean cover-glass, and examined with the 16-mm. objective, followed by the 4-mm. The oil-immersion lens should not be used for this purpose. It is convenient to handle the bits of sputum with a wooden toothpick or with a wooden cotton-applicator, which may be burned when done with. The platinum wire used in bacteriologic work is unsatisfactory because not usually stiff enough. A little practice is necessary before one can handle particles of sputum readily. The bit desired should be separated from the bulk of the sputum by cutting it free with the toothpick and drawing it out upon the dry portion of the glass dish. It can then be picked up by rotating the end of a fresh toothpick against it. *The slide must never be dipped into the sputum, nor must any of the sputum be allowed to run over its edges in spreading.*

The more important structures to be seen in unstained sputum are: elastic fibers, Curschmann's spirals, Charcot-Leyden crystals, pigmented cells, myelin globules, the ray fungus of actinomycosis, and molds. Forming the background for these are usually pus-corpuscles, granular detritus, and mucus in the form of translucent, finely fibrillar, or jelly-like masses. The pus-cells appear as finely granular grayish or yellowish balls, about 10 to 12 μ in diameter, and generally without visible nuclei (Figs. 24 and 25). They are best studied in stained preparations.

1. Elastic Fibers.—These are the elastic fibers of the pulmonary substance, where they are distributed in the walls of the alveoli, the bronchioles, and the blood-vessels. When found in the sputum they always indicate destructive disease of the lung, provided they do not come from the food, which is a not infrequent source. They are found in abscess and gangrene of the lung, but in the vast majority of cases their presence indicates tuberculosis. Advanced cases of tuberculosis often show great numbers, and, rarely, they may be found in early tuberculosis, when the bacilli cannot be detected. After the diagnosis is established they furnish a valuable clue as to the existence and rate of lung destruction.

In gangrene of the lung, contrary to the older teaching, elastic tissue is probably always present in the sputum, usually in large fragments.

The portion of sputum to be searched for elastic tissue should be selected by careful inspection. Small bits of necrotic tissue, or yellowish, greenish, or rusty particles, which are often minute, are most favorable; when these are absent the most purulent portion of the sputum should be taken. The selected bit is taken on a slide, and a cover-glass is applied and pressed down so as to give a moderately thin preparation, which is examined before it dries. Careful selection of the portion examined is more efficient than is the concentration method—boiling with 10 per cent. caustic soda and centrifugalizing—which is widely recommended.

FIG. 18.—Elastic fibers in tuberculous sputum, unstained, as seen with a low-power objective (× 100).

The search should be conducted with the 16-mm. objective, although a higher power is often needed to identify the fibers with certainty. They are slender, highly refractive, wavy fibrils with double contour and curled, often split, ends. Very characteristic are their graceful curves without sharp bends, their uniform diameter, and their smoothness, although in old sputum they may become much roughened. The fibers may lie singly or in bundles. Frequently they are found in alveolar arrangement, preserving the original outline of the alveoli of the lung (Figs. 18 and 19). This arrangement is positive proof of their origin in the lung.

Leptotrichia buccalis, which is a normal inhabitant of the mouth, may easily be mistaken for elastic tissue with the low power. It can usually easily be distinguished when studied with the 4-mm. objective. In case of doubt the iodin reaction (page 543) may be

tried, although not all forms of leptothrix react characteristically. Fatty acid crystals, which are often present in Dittrich's plugs and in sputum which has lain in the body for some time, also simulate elastic tissue when very long, but they are more like stiff, straight or curved needles than wavy threads. They show varicosities when the cover-glass is pressed upon and melt into droplets when the slide is heated. The structures which most frequently confuse the student are the cotton fibrils, which are often present as a contamination from the air. These are usually coarser than elastic fibers and flat, with one or two twists, and often have longitudinal striations and frayed-out ends. The color, too, is somewhat dif-

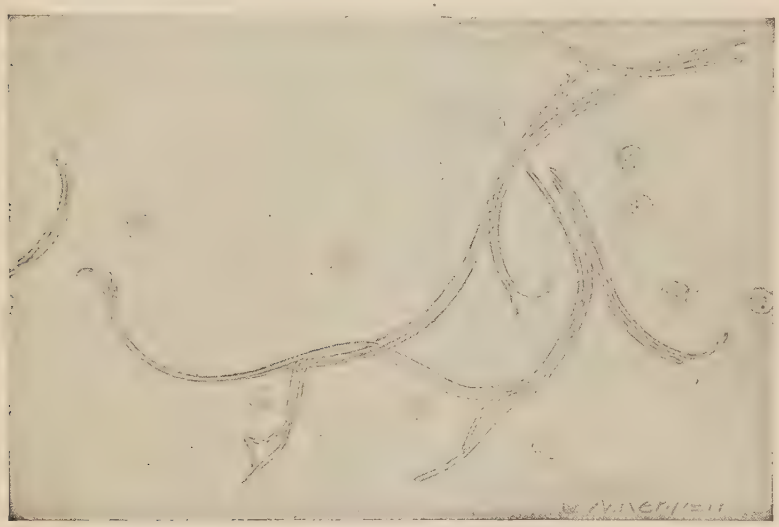


FIG. 19.—Elastic fibers in tuberculous sputum, unstained, as seen with a high-power objective (× 400).

ferent. Cotton fibers lack the faint yellowish tinge of elastic tissue. Very important also is the relative degree of refractility: if the diaphragm be opened slowly, elastic fibers can be seen long after the slightly refractile mucous strands have disappeared, but finally they also are practically lost in the glare, while cotton fibers still remain visible. In stained preparations students frequently report the fibrils of precipitated mucus as elastic tissue.

Elastic fibers from the food are coarser, generally shorter, less frequently wavy, and not arranged in alveolar order.

2. Curschmann's Spirals.—These peculiar structures are found most frequently in bronchial asthma, of which they are

fairly characteristic. Although not present in every attack, they probably occur at some time in every case. Sometimes they can be found only near the end of the attack. They may occasionally be met with in chronic bronchitis and other conditions, but in these there is nearly always an underlying asthmatic tendency. Their nature has not been definitely determined.

Macroscopically, they are whitish or yellow wavy threads, frequently coiled into little balls (Fig. 20). Their length is rarely over 1.5 cm., though it sometimes exceeds 5 cm. They can sometimes be definitely recognized with the naked eye. Under a 16-mm. objective they appear as mucous threads with a bright, colorless, central line—the so-called central fiber—about which are wound many fine fibrils (Figs. 21 and 22). The bright central line is best seen when the objective is raised a little above the true focus; and it has been interpreted as an optical phenomenon due to tight coiling of the spiral. In some cases one or more definite dark colored thread-like fibers can be seen at the true focus. The spiral fibrils are sometimes loosely, sometimes tightly wound. Eosinophils are usually present within them, and sometimes Charcot-Leyden crystals also. Not infrequently the spirals are imperfectly formed, consisting merely of twisted strands of mucus enclosing leukocytes. The central fiber is absent from these.

FIG. 20.—Curschmann's spirals in asthmatic sputum as seen when pressed out between two plates of glass and viewed over a black background. Each is embedded in a mass of grayish mucus. (Natural size.)

3. Charcot-Leyden Crystals.—Of the crystals which may be found in the sputum, the most interesting are the Charcot-Leyden crystals. They may be absent when the sputum is expectorated, and appear in large numbers after it has stood for some time. They are rarely found except in cases of bronchial asthma, and were at one time thought to be the cause of the disease. They frequently adhere to Curschmann spirals. Their exact nature is unknown. Their formation seems to be in some way connected with the presence of eosinophilic cells. Outside of the sputum they are found in the feces in association with animal parasites, and in the coagulated blood in leukemia.

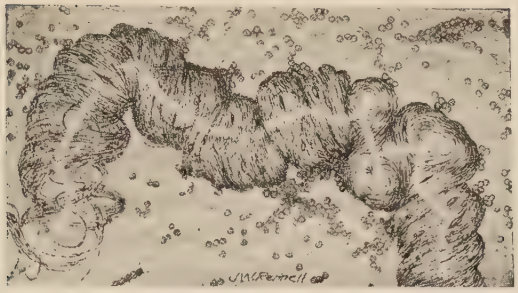


FIG. 21.—End of a large, tightly wound Curschmann's spiral in sputum from a case of bronchial asthma. Unstained (× 70).

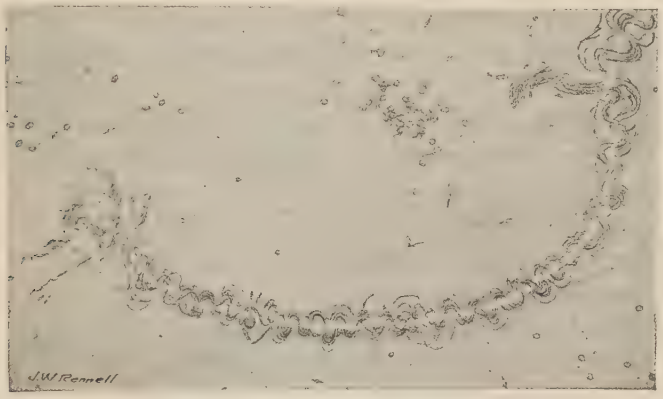


FIG. 22.—Slender, loosely wound Curschmann's spiral in sputum from a case of bronchial asthma. A few Charcot-Leyden crystals are also shown. Unstained (× 70).

FIG. 23.—Charcot-Leyden crystals and eosinophilic leukocytes in sputum from a case of bronchial asthma. Unstained (× 475). The magnification is greater than is usually used in studying these structures.

They are colorless, pointed, often needle-like crystals (Fig. 23). Formerly they were described as octahedral, but are now known to

be hexagonal in cross-section. Their size varies greatly, the average length being about three or four times the diameter of a red blood-corpuscle.

Other crystals—hematoidin, cholesterol, and, most frequently, fatty acid needles (Fig. 54)—are common in sputum which has remained in the body for a considerable time, as in abscess of the lung and bronchiectasis. The fatty acid crystals are regularly found in Dittrich's plugs. They might be mistaken for elastic fibers (p. 55). Sometimes they form rounded masses with the individual crystals radially arranged, and they then bear considerable resemblance to the clumps of *Actinomyces hominis*.

4. Pigmented Cells.—Granules of pigment are sometimes seen in ordinary pus-corpuscles, but the more common and important pigment-containing cells are large mononuclear cells whose origin is in some doubt. They were formerly thought to be the flattened epithelial cells which line the pulmonary alveoli. The present tendency is to identify them with the endothelial leukocytes, which are known to take up pigment granules readily; but in view of recent studies the question must be left open. Two kinds of pigmented cells deserve mention: those which contain blood-pigment, chiefly hemosiderin; and those which contain carbon. As a rule, these cells also have a coarsely granular appearance from the presence of many small colorless myelin globules.

To those which contain blood-pigment the name **heart-failure cells** has been given, because they are most frequently found in long-continued passive congestion of the lungs resulting from poorly compensated heart disease. The presence of these cells in considerable numbers, by directing one's attention to the heart, will sometimes clear up the etiology of a chronic bronchitis. They are sometimes so numerous as to give the sputum a brownish tinge. Such cells are also found in the sputum in pulmonary infarction and for some time after a pulmonary hemorrhage. In fresh unstained sputum heart-failure cells appear as round, grayish, or colorless bodies filled with variously sized rounded granules of yellow to brown pigment (Plate II, Fig. 1). Sometimes the pigmentation takes the form of a diffuse staining. The nucleus is usually obscured by the pigment. The cells are large, averaging about 35 μ in diameter.

To demonstrate the nature of the brown pigment apply a 10 per cent. solution of potassium ferrocyanid for a few minutes and follow with weak hydrochloric acid. Iron-containing pigment assumes a blue color. Many of the granules, will, however, fail to respond. The test may be applied either to wet preparations or to dried smears.

Carbon-laden cells (Plate II, Fig. 1) are less important. They are especially abundant in the sputum of anthracosis where angular black granules, both intracellular and extracellular, may be so numerous as to color the sputum. Similar cells with smaller carbon particles are often abundant in the morning sputum of those who inhale tobacco smoke to excess, or those who live in a smoky atmosphere.

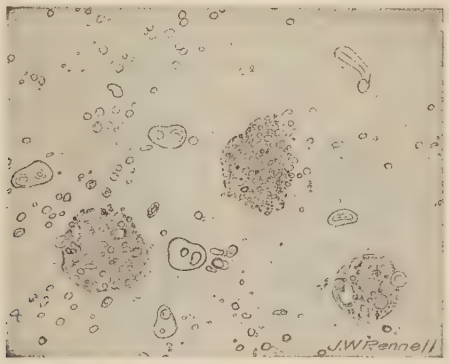


FIG. 24.—Myelin globules, free and contained within cells. From a "normal morning sputum (× 350).

5. Myelin Globules.—These have little or no clinical significance, but require mention because of the danger of confusing them with more important structures, notably blastomyces. They are colorless, round, oval, or pear-shaped globules of various sizes, often resembling fat-droplets, but the larger ones more frequently showing peculiar concentric or irregularly spiral markings (Figs. 24 and 32). Such globules are abundant in the scanty morning sputum of apparently healthy persons, but may be found in any mucoid sputum. They lie both free in the sputum and contained within the large cells which have long been known as alveolar cells, but which are possibly endothelial leukocytes. The intracellular globules are generally small, and when closely packed give the cells a yellowish tinge which may mislead the unwary into calling them heart-failure cells.

6. Actinomyces Hominis (Ray-fungus).—In the sputum of pulmonary actinomycosis and in the pus from actinomycotic lesions elsewhere, small gray or yellowish, "sulphur" granules can be detected with the unaided eye. Without a careful macroscopic examination they are almost certain to be overlooked. The fungus can be seen by crushing one of these granules between slide and cover, and examining with a low power. *Actinomyces hominis* is very similar to, and perhaps identical with, *Actinomyces bovis*, the cause of "lumpy-jaw" in cattle. It consists of a network of threads having a more or less radial arrangement (Figs. 25 and 26). In cattle, and to a less extent in man, the filaments at the periphery of the nodule present club-shaped extremities. It can be brought out more clearly by running a little solution of eosin in alcohol

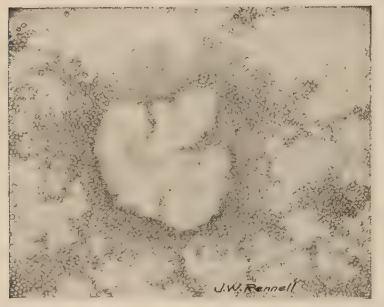


FIG. 25.—A "sulphur granule" crushed beneath the cover-glass. From the pus of a case of actinomycosis of submaxillary lymph-nodes. Unstained (× 60).

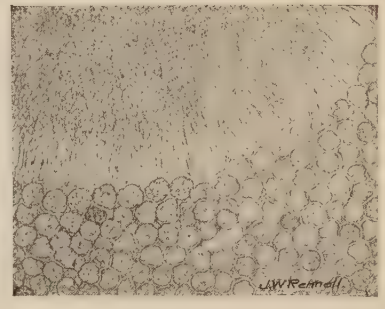


FIG. 26.—A portion of Fig. 25, more highly magnified (× 300).

and glycerin under the cover. This organism apparently stands midway between the bacteria and the molds and may be classed with the Trichomycetes or higher bacteria (p. 75). It stains by Gram's method.

Actinomycosis of the lung is rare. The clinical picture is that of tuberculosis.

7. Molds and Yeasts.—The hyphæ and spores of various molds are occasionally met with in the sputum. The hyphæ are rods, usually jointed or branches (Fig. 88), and often arranged in a meshwork (mycelium); the spores are highly refractive spheres and ovoids. Both stain well with the ordinary stains. Molds in the sputum are usually the result of contamination and have little significance. Occasionally they grow in the pus of lung cavities

whether of tuberculous or other origin. Very rarely there may be true infection of the lung—a pneumomycosis—usually resembling tuberculosis and most often due to *Aspergillus fumigatus*. Bronchopulmonary mycosis is said to be common in the Orient.

In the extremely rare condition of pulmonary blastomycosis specific yeasts have been found in the sputum in large numbers. In the tissues they multiply by budding and the presence of budding forms in the sputum is sufficient for their identification as blastomyces. In cultures they form hyphæ. The similar organism, *Coccidioides immitis*, the cause of coccidioidal granuloma of the Pacific Coast, does not form buds, but multiplies by endosporulation. For both these organisms it is advisable to add a little 10 per cent. caustic soda solution and examine unstained. Both may also be studied in stained smears, but cultural methods are essential for their complete identification.

As a contamination one sometimes finds forms of the fungus *Mycoderma*. These have a striking appearance, resembling gigantic sausage-shaped bacilli.

8. Animal Parasites.—These are extremely rare in the sputum in this country. A trichomonad, perhaps identical with *Trichomonas hominis*, has been seen in the sputum of putrid bronchitis and gangrene of the lung, but its causal relationship is doubtful. In Japan infection with the lung flukeworm, *Paragonimus westermanii*, is common, and the ova are found in the sputum. The lung is not an uncommon seat for echinococcus cysts, and hooklets and scolices may appear. Larvæ of *Strongyloides stercoralis* and of the hookworm have been reported. *Endamœba histolytica* has been found after rupture of an hepatic abscess into the lung. "Spirochetes," which are best studied in stained preparations, are described on page 76. Ciliated body cells, with cilia in active motion, are not infrequently seen, and may easily be mistaken for infusoria. All the above-mentioned parasites are described in Chapter VI.

B. Stained Sputum

The principal structures which are best seen in stained sputum are bacteria and cells.

A number of smears should be made upon slides or covers. These films must, of course, be thin, but it is easily possible to get them too thin. This is a common error of students who have just

finished a course in bacteriology and who have there been accustomed to work with scarcely perceptible films of bacteria. It is a good plan to slide off the cover-glass from the preparation used for the unstained microscopic examination. If this is properly done, satisfactory smears will be left on both slide and cover. They are then dried in the air and fixed in the flame, as described on page 657, or better, by immersion for one or two minutes in pure wood alcohol or 1 per cent. solution of corrosive sublimate. Fixation will ordinarily kill the bacteria and the smears may be kept indefinitely; but smears on slides when fixed by heat are often not sterile, and should be handled accordingly. As a matter of routine one of the smears should be stained for the tubercle bacillus and one by Gram's method with a good counterstain (p. 657). These preparations will give a good idea of the various cells and bacteria present and may suggest further procedure.

1. Microörganisms.—Saprophytic bacteria from mouth contamination are frequently present in large numbers and will prove confusing to the inexperienced. The presence of squamous cells in their neighborhood will suggest their source. Among the pathogenic organisms are: tubercle bacilli; staphylococci and streptococci; pneumococci; bacilli of Friedländer; influenza bacilli, and *Micrococcus catarrhalis*. Of these, the tubercle bacillus is the only one whose recognition has great clinical value and the only one which is easily identified in stained smears. Their cultural characteristics are described in Chapter X. When cultures are to be made the teeth and tongue should be well cleaned with a sterile brush, the mouth should be well rinsed, and the sputum, preferably only one expectoration, should be expectorated directly into a dry, sterile, wide-mouthed bottle. This should be kept on ice. As soon as possible the most purulent portions should be picked out, washed in several changes of sterile salt solution, and planted upon appropriate media.

(1) **Tubercle Bacillus.**—The presence of tubercle bacilli may be taken as positive evidence of the existence of tuberculosis somewhere along the respiratory tract, most likely in the lung, but when only one or two are found on a slide the result should be confirmed by a second examination. There is always the rare possibility that bacilli from a previous examination may have clung to an imperfectly cleaned slide, that bacilli may have reached the sputum from

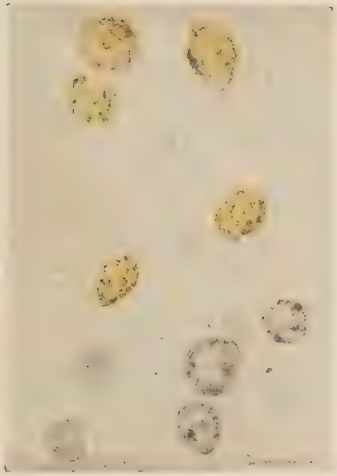


FIG. 1.—Heart-failure cells and carbon-laden cells in unstained sputum. Two small squamous epithelial cells and four red blood-corpuscles are included for comparison of size. (× 200.)

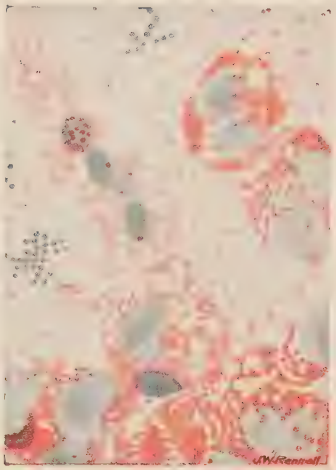


FIG. 2.—Eosinophilic leukocytes and staphylococci in asthmatic sputum. Eosin and methylene-blue. (× 1000.)

FIG. 3.—Tubercle bacilli, streptococci, pus corpuscles, and mucous threads in tuberculous sputum. Ziehl-Neelsen method. (× 1000.)

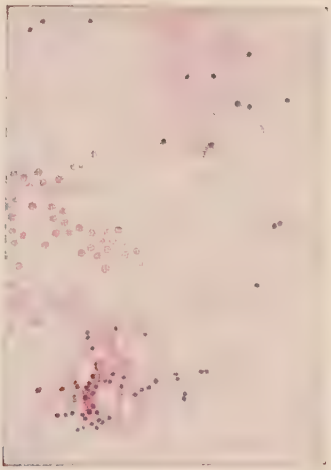


FIG. 4.—Much's granules from two fields of a slide stained as described in the text. A group of half-digested staphylococci is also shown. (× 1500.)

the dust of the air, or that the suspected bacillus may be some other acid-fast organism.

The importance of carefully selecting the portion for examination cannot be too strongly emphasized. It is always best to select the more purulent portions of the sputum, keeping away from the mucoid parts. If bits of necrotic tissue are present, they may show immense numbers of tubercle bacilli when other portions of the specimen contain very few. One must, however, be on his guard against bits of food which resemble these "caseous particles." The specimen should be examined while fresh. It will usually liquefy upon standing, and this, by preventing the selection of particles favorable for examination, will greatly reduce one's chances of finding bacilli.

Recognition of the tubercle bacillus depends upon the fact that it stains with difficulty; but that when once stained, it retains the stain tenaciously even when treated with a mineral acid, which quickly removes the stain from other bacteria. This "acid-fast" property is due to the presence of a waxy or lipoid substance. A number of the best staining methods are included here. Since Gabbet's method is convenient, inexpensive, and widely used in office work, it is given in greater detail than the others. If it is carried out as here recommended, there is no danger of decolorizing the tubercle bacillus. The author, however, would recommend Pappenheim's method for routine work, as least likely to give trouble to the inexperienced provided the solutions are good. Students rarely fail to get perfect results at the first trial. In large laboratories the Ziehl-Neelsen method is to be preferred.

Tubercle bacilli can often be found in very poorly prepared slides, but for dependable results when bacilli are scarce properly spread, fixed, and stained preparations free from precipitated stain are absolutely essential. *The person who is content with an imperfect preparation because it is "good enough for diagnosis" will succeed only in the most obvious cases.*

Gabbet's Method.—1. Spread suspicious particles thinly and evenly upon a slide or a cover-glass held in the grasp of cover-glass forceps. In general, slides are more satisfactory, but cover-glasses are easier to handle while staining. Do not grasp a cover too near the edge or the stain will not stay on it well. Tenacious sputum will spread better if gently warmed while spreading.

2. Dry the film in the air.

3. Fix the film by immersing in 1 per cent. aqueous solution of corrosive sublimate or in methyl alcohol for two or three minutes and then rinse well in water. This is much to be preferred, particularly for beginners, to the usual practice of fixing in the flame (p. 657). Should the film be washed off during future manipulations, fixation has been insufficient.

4. Apply as much carbolfuchsin (p. 697) as will stay on, and hold over a flame so that it will steam for three minutes or longer, replacing the stain with a dropper as it evaporates. If the stain be allowed to evaporate completely, the preparation is ruined. If the bacilli be well stained in this step, there will be little danger of decolorizing them later. Too great heat will interfere with the staining of some of the bacilli, probably by destroying the waxy substance upon which the acid-fast property depends. A number of slides may be stained at the same time by placing them upon a rack consisting of two parallel rods about 2 inches apart placed across a sink or on a tripod.

Recently it has been shown that twenty to thirty minutes' staining at room temperature will suffice, and this may be recommended on the score of avoiding precipitates, the slides being immersed in the fluid in a staining jar. With some batches of carbolfuchsin even five minutes' staining is sufficient.

5. Wash the film in water.

6. Apply Gabbet's stain (p. 698) to the under side of the cover-glass to remove excess of carbolfuchsin, and then to the film side. Allow this to act for one-quarter to one-half minute.

7. Wash in water.

8. If, now, the thinner portions of the film are blue, proceed to the next step; if they are still red, repeat steps 6 and 7 until the red has disappeared. Too long application of Gabbet's stain will decolorize the tubercle bacilli.

9. Place the preparation between layers of filter-paper and dry by rubbing with the fingers, as one would in blotting ink. Warm over the flame until thoroughly dry.

10. Put a drop of Canada balsam upon a clean slide, place the cover-glass film side down upon it, and examine with an immersion objective. Cedar oil or water may be used in place of balsam for temporary preparations. Smears on slides may be examined directly with an oil-immersion lens, no cover being necessary.

Ziehl-Neelsen Method.—The objection is often made to the above method that decolorization is masked by the blue in Gabbet's stain. Although this will not make trouble if step 8 is carefully carried out,

most experienced workers prefer the Ziehl-Neelsen method. This resembles Gabbet's method, with the following exceptions: After the staining with carbolfuchsin the smear is washed in 5 per cent. nitric acid (or, better, a mixture of 3 c.c. concentrated hydrochloric acid and 97 c.c. 70 per cent. alcohol) until only a faint pink remains in the thinner portions, washed in water, stained lightly with Löffler's methylene-blue, again washed, and finally dried and mounted. In place of methylene-blue, some prefer a counterstain consisting of equal parts of alcohol and saturated aqueous solution of picric acid. With this, tubercle bacilli stand out clearly against a faint yellow background, but non-acid-fast bacteria and cells are not recognizable.

Pappenheim's Method.—This is the same as Gabbet's method, except that Pappenheim's methylene-blue solution (p. 698) is substituted for Gabbet's stain and is allowed to act several times as long. To avoid evaporation the blue stain must be kept in a tightly corked bottle.

The method is very satisfactory for routine work. Decolorization of the tubercle bacillus is practically impossible: it retains its red color even when soaked overnight in Pappenheim's solution. The stain was originally recommended as a means of differentiating the smegma bacillus, which is decolorized by it, but it is not to be absolutely relied upon for this purpose.

In films stained by these methods tubercle bacilli, if present, will be seen as slender red rods upon a blue background of mucus, which appears as delicate threads and strands, granular detritus, and cells (Plate II, Fig. 3). They vary considerably in size, averaging 3 to 4 μ in length—about one-half the diameter of a red blood-corpuscle. Beginners must be warned against mistaking the edges of cells, or particles which have retained the red stain, for bacilli. The appearance of the bacilli is almost always typical, and if there seems room for doubt, the structure in question is probably not a tubercle bacillus. They may lie singly or in groups. They are very frequently bent and often have a beaded appearance. It is possible that the larger beaded bacilli indicate a less active tuberculous process than do the smaller uniformly stained ones. sometimes they are present in great numbers—thousands in a field of the 2-mm. objective. Sometimes, even in advanced cases, several slides must be examined to find a single bacillus. At times they are so few that none are found in stained smears, and special methods are required to detect them. The number may bear some relation

to the severity of the disease, but this relation is by no means constant. The mucoid sputum from an incipient case sometimes contains great numbers, while sputum from large tuberculous cavities at times contains very few. Failure to find them is not conclusive, though *their absence is much more significant when the sputum is purulent than when it is mucoid.*

The approximate number of bacilli present should always be indicated in the record of the examination. This may be done by recording the average number seen in a field. Since the sputum raised at various times in the day, and even different parts of the same sample, may vary greatly in bacillary content, such a record is not an accurate index of the comparative number of bacilli thrown off, even when the twenty-four-hour sputum is collected and uniformly mixed before preparing the slides. It is, however, a useful clinical guide.

When bacilli are not found in suspected cases, one of the following methods should be tried:

1. **Antiformin Method.**—This has largely superseded the older methods of concentration. The chief difficulty with the older methods, such as boiling with caustic soda, is that the bacilli are so injured in the process that they do not stain characteristically. Excepting in special cases, when the bacilli are both scarce and uniformly scattered through the sputum, it is doubtful whether any method of concentration offers any advantage over the usual direct smear made from carefully selected particles.

Antiformin is a trade name for a preparation consisting essentially of equal parts of a 15 per cent. solution of caustic soda and a 20 per cent. solution of sodium hypochlorite. Substitutes appear to be less satisfactory than the original preparation. The solution slowly loses strength upon standing.

Löffler's method is probably the best for clinical work. It kills the bacilli, so that there is no danger in handling the material. Upon this account, however, it is not applicable to isolation of tubercle bacilli for cultures.

Place 10 to 20 c.c. of the sputum in a small flask, with an equal amount of 50 per cent. antiformin, and heat to the boiling-point. The sputum will be thoroughly liquefied, usually within a few seconds. For each 10 c.c. of the resulting fluid add 1.5 c.c. of a mixture of 1 volume of chloroform and 9 volumes of alcohol. Insert a rubber stopper and shake vigorously for several minutes or until emulsification has taken

place. The object is to impregnate the lipoid capsule of the bacilli with chloroform, thus increasing their specific gravity. Pour off the emulsion into centrifuge tubes and centrifugalize at high speed for about fifteen minutes. The chloroform will go to the bottom, and the sediment which collects on its surface in a thin firm layer will contain the tubercle bacilli. Pour off the supernatant liquid and transfer the sediment to glass slides, removing the excess of fluid with filter-paper. To facilitate removal of the disk of sediment *in toto* Williamson recommends the use of a centrifuge tube, the lower $\frac{1}{2}$ inch of which is of uniform caliber and the bottom of which is open and plugged with a rubber stopper. Add a little of the original sputum to cause the film to adhere to the slide, mix well, spread into a uniform layer, and finally dry, fix, and stain by the Ziehl-Neelsen method. Löffler recommends 0.1 per cent. solution of malachite green for counterstain.

2. **Animal Inoculation.**—Inoculation of guinea-pigs is the court of last appeal in detection of tubercle bacilli, but even this is not infallible, for it has been shown that the injected material must contain 10 to 150 bacilli in order to produce tuberculosis in the guinea-pig, the number required depending upon the virulence. The method is described on page 541.

There are a number of bacilli which stain in the same way as the tubercle bacillus and, therefore, belong to the group known as **acid-fast bacilli.** They stain with difficulty, and when once stained give up the color only very slowly when treated with a mineral acid; but, unlike the tubercle bacillus, most of them can be decolorized with alcohol or with Pappenheim's solution. Among them are the leprosy, smegma, butter and grass bacilli, and a bacillus which has been found in old distilled water. For this reason old distilled water should never be used for rinsing films or making staining solutions. Of the acid-fast bacilli, the smegma bacillus is the only one likely to cause confusion. It occurs normally about the genitals and other parts of the body, as the axillæ, where secretions are prone to collect, and is often present in the urine and in the wax of the ear. It, or a similar bacillus, is sometimes found in the sputum of gangrene of the lung. The method of distinguishing it from the tubercle bacillus is given later (p. 200). A streptothrix (p. 76), which resists Gabbet's solution but is readily decolorized by Pappenheim's, has also been found in the sputum.

Other bacteria than the acid-fast group are stained blue by Gabbet's and the Ziehl-Neelsen method. Those most commonly

found are staphylococci, streptococci, and pneumococci. Their presence in company with the tubercle bacillus constitutes *mixed infection*, although it is doubtless true that some of them in many cases exist as saprophytes. It is to be remembered that a few of the bacteria may reach the sputum from the upper air-passages, and that great numbers are usually present in decomposing sputum.

There are many varieties of tubercle bacilli pathogenic for different animals, for example, the human, bovine, avian, and reptilian bacilli. Both human and bovine types are pathogenic for man, the latter being most frequently found in intestinal and lymphatic lesions of infancy and childhood. In clinical work it is not practicable to distinguish between the two.

Within the past few years much interest has centered in the so-called **"Much's granules."** These are Gram-positive, but non-acid-fast granules which are apparently forms of the tubercle bacillus, since material containing them causes tuberculosis when injected into guinea-pigs. They may be present either alone or in company with the ordinary acid-fast form.

It is now fairly well established that Much's granules represent a less virulent form of the tubercle bacillus which is especially frequent in quiescent and mild chronic cases, and that they give place to the acid-fast forms when such cases become active. Their detection is therefore important, but it is not easy because of other granules—precipitated stain, micrococci, etc.—which may be mistaken for the true Much bodies. The following method, while somewhat complicated, reduces the chance for error to the minimum.

Staining Method for Much's Granules.—1. To the twenty-four-hour amount of sputum add an equal volume of 0.6 per cent. sodium carbonate solution, shake thoroughly, and allow to stand in a warm place (preferably the incubator) for twenty-four hours. If it is not then completely homogeneous, extend the time to forty-eight hours.

2. Centrifugalize thoroughly, remove and discard half of the supernatant fluid, and mix an equal volume of 30 per cent. antiformin with the remaining half. Allow this to act for thirty minutes. It is imperative that the antiformin be fresh.

3. Centrifugalize, and make smears from the sediment. Centrifugation must be very thorough, otherwise the granules may remain sus-

pended. The specific gravity of the fluid may be reduced with alcohol if necessary. Fixation is not necessary if the films be carefully handled.

4. Stain one smear by the Ziehl-Neelsen method. This will demonstrate the ordinary tubercle bacilli, if present, and will also serve to show whether any cocci have been left undigested.

5. Immerse the remaining smears for forty-eight hours in a stain consisting of:

Carbolfuchsin	3 parts
Carbol-methyl violet	1 part

This stain remains good for about two weeks. The carbol-methyl violet used for this purpose consists of 2 per cent. phenol, 9 parts; saturated alcoholic solution of methyl violet, 1 part. In order to avoid precipitates slides should stand on edge while in the stain.

6. Rinse gently in water.

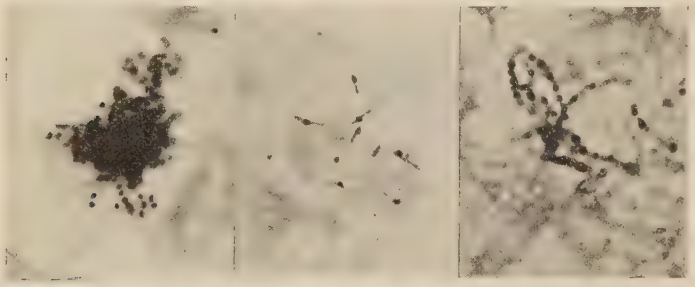


FIG. 27.—Much's granules in sputum stained by the method detailed in the text. Note the different modes of grouping (photographs × 1500).

7. Cover with Gram's iodin solution for five minutes, warming until steam rises.

8. Decolorize successively with 5 per cent. nitric acid for one minute, with 3 per cent. hydrochloric acid for ten seconds, and finally with a mixture of equal parts of acetone and 95 per cent. alcohol until color ceases to come off.

9. Dry, mount in cedar oil or balsam, and examine with the oil-immersion lens.

Steps 1 and 2 in this method serve the double purpose of concentrating the sputum and of digesting any micrococci which may be present and which might be confused with Much's granules. One must be cautious in interpreting isolated granules if any undigested cocci are found in the control slide. Partially digested cocci which take the color of the background will not cause confusion. The concentration and digestion may be omitted if desired, but results are then much less dependable.

Much's granules (Plate II, Fig. 4) are definite, clean-cut, round, or oval bodies about 0.5 μ in diameter. They are thus about half the diameter of a staphylococcus. Ordinarily they are deep purple, often with a tinge of red. They may lie singly or, more frequently, in rows of two to five, or in clusters (Fig. 27). Connecting the granules can usually be seen a faint bluish or reddish band suggesting the body of a bacillus in or on which the granules lie. Isolated granules usually appear to lie at the end or in the middle of such a band, and unless the band is seen they should not be accepted as true Much's granules.

(2) **Staphylococcus and Streptococcus.**—One or both of these organisms is commonly present in company with the tubercle bacillus in the sputum of advanced phthisis (Plate II, Figs. 2 and 3). They are often found in bronchitis, catarrhal pneumonia, and many other conditions. The streptococcus is much the more important. It is a common cause of severe sore throat and tonsillitis. Staphylococci and streptococci are sometimes found in sputum and normal mouths as saprophytes. These cocci are discussed more fully on page 523, and their cultural characteristics are described on page 663.

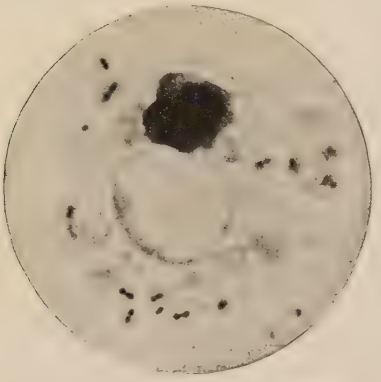


FIG. 28.—Pneumococci in exudate from human lung (Kolle and Wassermann).

(3) **Pneumococcus (Diplococcus of Fränkel).**—The pneumococcus is the causative agent in nearly all cases of croupous pneumonia, and is commonly found in large numbers in the rusty sputum of this disease. It is frequently met with in the sputum of catarrhal pneumonia, bronchitis, and tuberculosis, although here it is not infrequently a harmless saprophyte. It is also an important factor in the causation of pleurisy, meningitis, otitis media, and other inflammations. It is frequently present in the saliva in health.

Pneumococci are about the size of streptococci. They are ovoid in shape, and lie in pairs, end to end, often forming short chains. Each pair is surrounded by a gelatinous capsule, which is its distinctive feature (Fig. 28).

The pneumococcus is closely related to the streptococcus, and it is

sometimes extremely difficult to differentiate them even by cultural methods (p. 663). The morphology of the pneumococcus, the fact that it is Gram-positive, and the presence of a capsule are, however, generally sufficient for its recognition in smears from sputum or pus. The capsule is often seen as a halo around pairs of cocci in smears stained by the ordinary methods, particularly Gram's method, but to show it well special methods are required. There are numerous special methods of staining capsules, which are applicable to other encapsulated bacteria, as well as to the pneumococcus. Smith's and Rosenow's methods, described below, and Hiss's method, described on page 523, can be recommended; but any of the standard methods will give beautiful results if capsules are well developed. The chief requisite is that the sputum or pus be very fresh—not more than two or three hours old.

W. H. Smith's Method for Capsules.—1. Make thin smears of the sputum or other material, which should be as fresh as possible.

2. Fix in the flame in the usual manner.

3. Apply a 10 per cent. aqueous solution of phosphomolybdic acid (Merck) for four to five seconds.

4. Rinse in water.

5. Apply anilin-gentian violet (p. 697), steaming gently for fifteen to thirty seconds.

6. Rinse in water.

7. Apply Gram's iodin solution, steaming gently for fifteen to thirty seconds.

8. Wash in 95 per cent. alcohol until the purple color ceases to come off.

9. Rinse in water.

10. Apply a 6 per cent. aqueous solution of eosin and gently warm for one-half to one minute.

11. Rinse in water.

12. Wash in absolute alcohol.

13. Clear in xylol.

14. Mount in balsam.

This is essentially Gram's method (p. 657), preceded by treatment with phosphomolybdic acid and followed by eosin. Gram-positive bacteria like the pneumococcus are deep purple; capsules are pink and stand out clearly.

When the method is applied to Gram-negative bacteria steps 5 to 9 inclusive are omitted; and between steps 11 and 12 the preparation is

counterstained with Löffler's methylene-blue, gently warming for fifteen to thirty seconds.

Rosenow's Method.—This is the same as Smith's, with the exception that a 10 per cent. solution of tannic acid, applied while the film is still moist and allowed to act for ten to twenty seconds, takes the place of the heat and phosphomolybdic acid in steps 2 and 3.

The cultural characteristics of the pneumococcus are described on page 665. By means of certain immunologic reactions it can be shown that there are at least four distinct types. These differ in their virulence. For organisms of Type I (present in about one-third of the cases of croupous pneumonia) an antiserum, which is valuable in treatment of this type of infection, has been prepared. Methods of determining pneumococcus types, either by agglutinins or precipitins, are described on page 666.

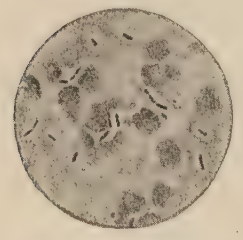


FIG. 29. — Friedländer's bacillus in pus from pulmonary abscess (× about 1000). (Boston.)

(4) **Bacillus of Friedländer (Bacillus mucosus capsulatus).**—In a small percentage of cases of pneumonia this organism is found alone or in company with the pneumococcus. Its pathologic significance is uncertain. It is often present in the respiratory tract under normal conditions. Friedländer's bacilli are non-motile, encapsulated rods, sometimes arranged in short chains (Fig. 29). Very short individuals in pairs closely resemble pneumococci, from which they are distinguished by the fact that they are Gram-decolorizing.

(5) **Bacillus of Influenza**—For many years this organism was regarded as the cause of true influenza, although it was recognized that clinically similar or identical conditions could be produced by the pneumococcus and streptococcus. It is present, sometimes in great numbers, in the nasal and bronchial secretions of most cases of influenza and in the local lesions which follow as complications. Chronic bronchitis, clinically suggesting tuberculosis, but apparently of influenzal origin, with great numbers of the bacilli in the sputum, has been described.

The many studies during the great pandemic of 1918-19 have, however, failed to confirm the specific importance of the influenza

bacillus. It seems probable that it is not the primary cause of influenza; that in most cases it is merely a secondary invader, although it may be extremely important even in that rôle. The question remains unsettled.

Recognition of influenza bacilli in smears depends on the facts that they are extremely small bacilli; that most of them lie within the pus-cells; that their ends often stain more deeply than their centers, sometimes giving the appearance of minute diplococci; and that they are decolorized by Gram's method of staining (Figs. 30 and 267).

They are well stained by dilute fuchsin or by Pappenheim's pyronin-methyl green, but are more certainly recognized by Gram's method with the pyronin-methyl green for counterstain.

(6) **Bacillus Pertussis.**—The whooping-cough bacillus, which is found in the sputum in large numbers early in the disease, is a minute, ovoid, Gram-negative bacillus which stains feebly with the ordinary dyes, and sometimes, though not usually, lies within pus-cells. It can be demonstrated by the method given for the influenza bacillus which it resembles morphologically.

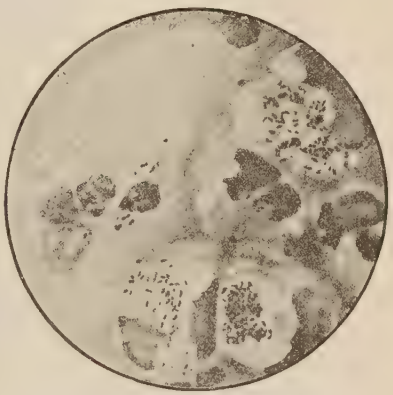


FIG. 30.—Bacillus of influenza; cover-glass preparation of sputum from a case of influenza, showing the bacilli in leukocytes; highly magnified (Pfeiffer).

(7) **Micrococcus Catarrhalis.**—This organism is frequently present in the sputum in inflammatory conditions of the respiratory tract resembling influenza. It is sometimes present in the nasal secretions in health and is probably of very little pathogenic significance. It is a Gram-negative diplococcus, frequently intracellular, and can be distinguished from the meningococcus and gonococcus only by means of cultures (Fig. 31). The staining method recommended for the influenza bacillus is best. It grows readily on ordinary media.

(8) **Higher Bacteria.**—Standing between the bacteria and the molds is a group of higher bacteria at one time called Trichomycetes or "hair fungi." They all form filaments, which are more slender

than the hyphæ of molds. Their classification is somewhat unsettled. Buchanan, Bergey, and the Society of American Bacteriologists now include them in the order of Actinomycetales, and in the family of Actinomycetaceæ. The genera Leptotrichia and Actinomyces, as well as two others not found in sputum, are in the family. *Leptotrichia buccalis* forms long, unbranched filaments, with partition walls. Its normal habitat is the oral cavity. There are numerous species of the genus Actinomyces. They may be aërobic, or anaërobic, acid-fast, or non-acid-fast. The most common forms found in sputum are *Actinomyces hominis* (p. 62) and *Actinomyces farcinicus* (also known as Nocardia, and also as Streptothrix). This last named species forms filaments, but no "clubs," is aërobic, and produces yellowish-white pigment on agar or potato medium. It is moderately acid-fast, but may be partially decolorized by alcohol and readily by Pappenheim's solution. In the sputum it appears in the form of short, very slender, branching filaments which usually lie in small tangled clusters that might be mistaken for clumps of tubercle bacilli by the inexperienced.

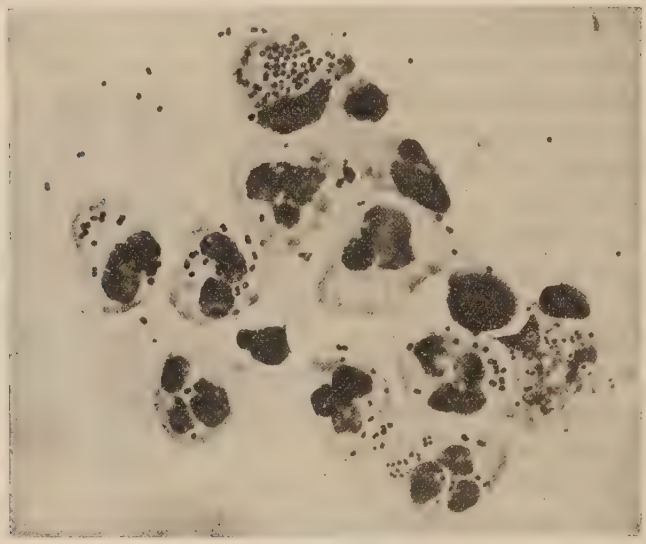


FIG. 31.—*Micrococcus catarrhalis* in smear from sputum (F. T. Lord; photo, by L. S. Brown).

(9) **Spirochetes.**—Since the first recognition of bronchial spirochetosis and description of *Spirochæta bronchialis* in 1906 by Castellani cases have been reported in nearly all tropical and sub-

tropical countries, in Southern Europe, and more recently in the United States.[1]

The cases have resembled tuberculosis clinically, with hemoptysis a frequent symptom.

The sputum should be as fresh as possible, as *Spirochæta bronchialis* soon breaks up into granules. Smears are made in the ordinary way and stained by any good spirochetal stain such as those given for *Treponema pallidum* (p. 558). In the method used for the tubercle bacillus and in Gram's method these spirochetes stain only lightly or not at all; hence they will be overlooked in the routine examination. They are generally present in large numbers, one to many in a field. They are described on page 460. In view of the prevalence of spirochetes in the mouth, no significance should be attached to isolated spirochetes in the sputum. In certain cases of pulmonary gangrene spirochetes and fusiform bacilli of the Vincent type (p. 460) have been found in large numbers and have apparently played a causative rôle. In these cases there were usually also mouth lesions harboring the same organisms.

2. Cells.—These include various types of leukocytes, epithelial cells, and red blood-corpuscles. In general, a stain of the nature of Wright's blood-stain is most satisfactory.

(1) **Leukocytes.**—(*a*) *Polymorphonuclear neutrophils* are present as pus-corpuscles in every sputum, and at times the sputum may consist of little else. They appear as granular, rounded cells 10 to 12 μ in diameter, with several nuclei or one very irregular nucleus which when unstained are obscured by the granules. In preparations stained by any of the usual methods the nuclei stand out clearly and their polymorphous character makes identification of these cells easy (Plate II, Fig. 3). In old sputa the cells may be much disintegrated and hence difficult to recognize even when stained. When these cells predominate in the sputum a pyogenic infection may be assumed.

(*b*) *Lymphocytes* are generally present in small numbers along with the ordinary pus-corpuscles, from which they are distinguished by the possession of a single round nucleus. In early or mild cases of pure tuberculous infection they are usually the pre-

[1] Levy, M. D.: Bronchopulmonary spirochetosis, New York Med. Jour., vol. 113, p. 186, January 29, 1921. Bloedorn, W. A., and Houghton, J. E.: Bronchial spirochetosis, Jour. Amer. Med. Assoc., vol. 76, p. 1559, June 4, 1921.

dominating cell, and may be of much help in distinguishing such cases from those due to pyogenic organisms. If, in a case of known tuberculosis, the "cell formula" changes from lymphocytic to polynuclear, the occurrence of a secondary infection is strongly suggested.

(*c*) *Eosinophilic leukocytes* are quite constantly found in large numbers in the sputum of bronchial asthma near the time of the paroxysm, and constitute one of the most distinctive features of the sputum of this disease. However, while of much diagnostic importance, they are by no means pathognomonic of asthma. They resemble ordinary pus-corpuscles, except that their cytoplasm is filled with coarse granules having a marked affinity for eosin. It is worthy of note that sometimes many of them are mononuclear. These are involution forms and not myelocytes. The eosinophils are very fragile; and large numbers of free granules, derived from disintegrated cells, are also found (Plate II, Fig. 2). Eosinophils can often be recognized in unstained sputum by the coarseness of their granules (Fig. 23), but for positive identification some method which includes eosin must be used. A simple method is to stain the dried and fixed film two or three minutes with saturated solution of eosin, and then with Löffler's methylene-blue for one-half minute or until the thinner portions of the film become blue; nuclei and bacteria will be blue, eosinophilic granules, bright red. Either Wright's or Jenner's stain (pp. 272 and 275) will also be found satisfactory.

(*d*) *Endothelial leukocytes* are best studied in unstained sputum, and have been described in the sections upon Pigmented Cells and Myelin Globules (pp. 60 and 61).

(2) **Epithelial cells** may come from any part of the respiratory tract. A few are always present, since desquamation of cells goes on constantly. Their recognition is important, chiefly as an aid in deciding upon the source of the portion of the sputum in which they are found. For this reason they are sometimes spoken of as "guide cells." In suspected lung conditions it is manifestly useless to study material from the nose only, yet this is not infrequently done. They have little diagnostic value, although a considerable excess would indicate a pathologic condition at the site of their origin. Any of the stains mentioned above will show them, and they can usually be identified without difficulty in unstained sputum. In general, three forms are found:

(*a*) *Squamous Cells.*—Large, flat, polygonal cells with a comparatively small nucleus (Fig. 32, *i*). They come from the upper air-passages, and are especially numerous in laryngitis and pharyngitis. They are frequently studded with bacteria—most commonly diplococci.

(*b*) *Cylindric Cells from the Nose, Trachea, and Bronchi* (Fig. 32, *f*, *h*).—These are not usually abundant, and, as a rule, they are not identified because much altered from their original form, being usually round and swollen. Cylindric cells with cilia intact are rare, but are sometimes seen in bronchial asthma and very acute

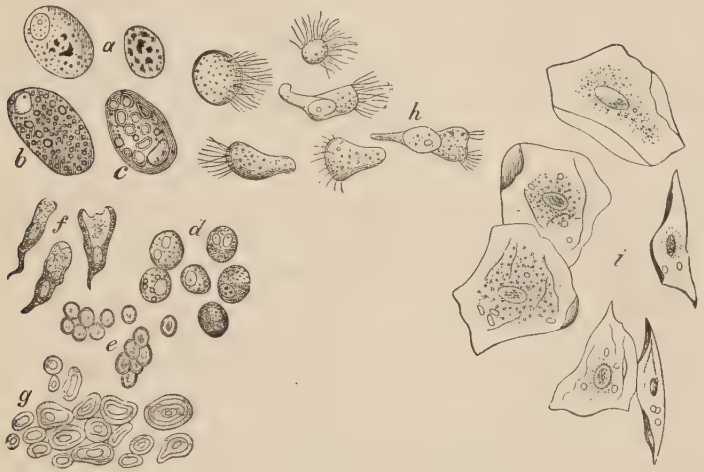


FIG. 32.—Different morphologic elements of the sputum (unstained): *a*, *b*, *c*, Pulmonary or alveolar epithelium—*a*, with normal lung pigment (carbon); *b*, with fat-droplets; *c*, with myelin globules; *d*, pus-corpuscles; *e*, red blood-corpuscles; *f*, cylindric beaker-shaped bronchial epithelial cells; *g*, free myelin globules; *h*, ciliated epithelium of different kinds from the nose, altered by coryza; *i*, squamous cells from the pharynx. (After Bizzozero.)

bronchitis. When very fresh the cilia may still be in active motion, suggesting infusoria.

(*c*) *Alveolar Cells.*—Rather large, round, or oval cells, three to six times the diameter of a red corpuscle, with one or two round nuclei (Fig. 32). Their source is presumably the pulmonary alveoli. It is probable that many of the cells which have been included in this group are really endothelial leukocytes.

(3) **Red blood-corpuscles** may be present in small numbers in almost any sputum. When fairly constantly present in considerable numbers they are suggestive of phthisis. The corpuscles,

when fresh, can easily be recognized in unstained sputum, or may be shown by any of the staining methods which include eosin. They are, however, commonly so much degenerated as to be unrecognizable, and often only altered blood-pigment is left. Ordinarily, blood in the sputum is sufficiently recognized with the naked eye.

III. CHEMICAL EXAMINATION

There is little to be learned from a chemical examination, and it is rarely undertaken. The presence or absence of albumin may have clinical significance. Albumin is almost constantly present in the sputum in pneumonia, pulmonary edema, and tuberculosis. It is usually absent in bronchitis. A test for albumin may, therefore, be of some value in distinguishing between bronchitis and tuberculosis, but it is not much relied upon. It is carried out as follows:

Method for Albumin in Sputum.—1. To 10 c.c. of the sputum add 30 c.c. of 1 per cent. acetic acid and shake until thoroughly mixed. This may be done in a stoppered bottle. Dilution and addition of acetic acid precipitates the mucus.

2. Filter through filter-paper.

3. Test the filtrate for albumin qualitatively and quantitatively, as described in Chapter II.

Active cases of phthisis, whether early or far advanced, generally show 0.2 per cent. or more albumin; slightly active cases, less than 0.2 per cent. The sputum must be fresh, otherwise a negative reaction may have changed to positive owing to disintegration of cells.

IV. THE SPUTUM IN DISEASE

Strictly speaking, any appreciable amount of sputum is abnormal. A great many healthy persons, however, raise a small quantity each morning, owing chiefly to the irritation of inhaled dust and smoke. Although not normal, this can hardly be spoken of as pathologic. Ordinarily it reaches the larynx without cough. It is particularly frequent in city dwellers and in those who smoke cigarettes to excess. In the latter the amount is sometimes so great as to arouse suspicion of tuberculosis. Such "normal morning sputum" or "sputum of irritation" generally consists of small,

rather dense, mucoid masses, translucent, white, or, when due to inhaled smoke, gray in color. Microscopically, there are a few pus-corpuscles, and, usually, many endothelial leukocytes, both of which may contain carbon particles. The endothelial leukocytes commonly show myelin degeneration, and free myelin globules may be present in large numbers. Saprophytic bacteria may be present, but are not abundant.

1. Acute Bronchitis.—There is at first a small amount of tenacious, almost purely mucoid sputum, frequently blood streaked. This gradually becomes more abundant, mucopurulent in character, and yellowish or gray in color. At first the microscope shows a few leukocytes and bronchial cells; later the leukocytes become more numerous. Bacteria are not usually abundant.

2. Chronic Bronchitis.—The sputum is usually abundant, mucopurulent, and yellowish or yellowish-green in color. Nummular masses like those of tuberculosis are sometimes seen. Microscopically, there are great numbers of pus-corpuscles, often much disintegrated. Epithelium is not abundant. Bacteria of various kinds, especially staphylococci, are usually numerous.

In fibrinous bronchitis there are found, in addition, fibrinous casts, usually of medium size.

In the chronic bronchitis accompanying long-continued passive congestion of the lungs, as in poorly compensated heart disease, the sputum may assume a rusty brown color, owing to presence of large numbers of the "heart-failure cells" previously mentioned.

3. Bronchiectasis.—The characteristic sputum is greenish or grayish, purulent, very abundant—sometimes as much as a liter in twenty-four hours -and has an offensive odor. It is thinner than that of chronic bronchitis, and upon standing separates into three layers of pus, serum, and frothy mucus. It contains great numbers of miscellaneous bacteria. Small hemorrhages are common. A feature of cases with a single large cavity is the periodic emptying of the cavity, usually upon rising in the morning; in other cases no periodicity is evident.

4. Gangrene of the Lung.—The sputum is abundant, fluid, very offensive, and brownish in color. It separates sharply into three layers upon standing—a thick brownish deposit of pus, débris, and blood-pigment, a clear fluid, and a frothy layer. Mi-

croscopically, few cells of any kind are found. Bacteria are extremely numerous; among them may sometimes be found an acid-fast bacillus probably identical with the smegma bacillus. As before stated, elastic fibers are usually present in large fragments.

5. Pulmonary Edema.—Here there is an abundant, watery, frothy sputum, varying from faintly yellow or pink to dark brown in color; a few leukocytes and epithelial cells and varying numbers of red blood-corpuscles are found with the microscope.

6. Bronchial Asthma.—The sputum during and following an attack is scanty, mucoid, and very tenacious. Most characteristic is the presence of Curschmann's spirals, Charcot-Leyden crystals, and eosinophilic leukocytes.

7. Croupous Pneumonia.—Characteristic of this disease is a scanty, rusty red, very tenacious sputum, containing red corpuscles or altered blood-pigment, leukocytes, epithelial cells, usually many pneumococci, and often very small fibrinous casts. This sputum is seen during the stage of red hepatization. During resolution the sputum assumes the appearance of that of chronic bronchitis. When pneumonia occurs during the course of a chronic bronchitis, the characteristic rusty red sputum may not appear.

8. Pulmonary Tuberculosis.—The sputum is variable. In the earliest stages it may appear only in the morning, and is then scanty and almost purely mucoid, with an occasional yellow flake; or there may be only one small mucopurulent mass no larger than a match head. When the quantity is small there may be no cough, the sputum reaching the larynx by action of the bronchial cilia. This is not well enough recognized by practitioners. A careful inspection of all the sputum brought up by the patient on several successive days, and a microscopic examination of all yellow portions, will not infrequently establish a diagnosis of tuberculosis when physical signs are negative. Intelligent co-operation of the patient is essential in such cases. Tubercle bacilli will sometimes be found in large numbers at this stage. Blood-streaked sputum is strongly suggestive of tuberculosis, and is more common in the early stages than later. It usually indicates an advancing process.

The sputum of more advanced cases resembles that of chronic bronchitis, with the addition of tubercle bacilli and elastic fibers. Nummular masses—circular, "coin-like" disks, which sink in water—may be seen. Caseous particles containing immense num-

bers of the bacilli are common. Far-advanced cases with old cavities often show rather firm, spheric or ovoid grayish masses in thin fluid—the so-called "globular sputum." These globular masses usually contain many tubercle bacilli. Considerable hemorrhages are not infrequent, and for some time thereafter the sputum may contain clots of blood or be colored brown.

CHAPTER II

THE URINE

Preliminary Considerations.—The urine is an extremely complex aqueous solution of various organic and inorganic substances. Most of the substances are either waste-products from the body metabolism or products derived directly from the foods eaten. Normally, the total amount of solid constituents carried off in twenty-four hours is about 60 gm., of which the organic substances make up about 35 gm. and the inorganic about 25 gm.

The most important organic constituents are urea, uric acid, and creatinin. Urea constitutes about one-half of all the solids, or about 30 gm., in twenty-four hours.

The chief inorganic constituents are the chlorids, phosphates, sulphates, and ammonia. The chlorids, practically all in the form of sodium chlorid, make up about one-half of the inorganic substances, or about 13 gm., in twenty-four hours.

Certain substances appear in the urine only in pathologic conditions. The most important of these are proteins, sugars, acetone and related substances, bile, and hemoglobin.

In addition to the substances in solution all urines contain various microscopic structures.

While, under ordinary conditions, the composition of urine does not vary much from day to day, it varies greatly at different hours of the same day. It is evident, therefore, that the **collection of the specimen** is important, and that *no quantitative test can be of value unless a sample of the mixed twenty-four-hour urine be used.* The patient should be instructed to void all the urine during the twenty-four hours into a clean vessel kept in a cool place, to mix it well, to measure the whole quantity, and to bring 8 or more ounces for examination. In order to avoid annoying misunderstandings it is well to make these directions specific, telling him to empty the bladder at a specified time, say 8 A. M., and to discard this urine, to save all the urine voided up to 8 A. M. of the next day, and at that time to empty the bladder whether he feels the need for it or not, and to add this final amount to the quantity collected.

When it is desired to make only qualitative tests, as for albumin or sugar, a "sample" voided at random will answer. It should be remembered, however, that urine passed about three hours after a meal is most likely to contain pathologic substances. That voided first in the morning is least likely to contain them. To diagnose cyclic albuminuria samples obtained at various periods during the twenty-four hours must be examined.

The urine must be examined while fresh. **Decomposition** sets in rapidly, especially in warm weather, and greatly interferes with all the examinations. Decomposition may be delayed by adding 5 grains of boric acid (as much of the powder as can be heaped upon a 10-cent piece) for each 4 ounces of urine, but this causes precipitation of rhombic crystals of uric acid and does not prevent the growth of yeast. Formalin, in proportion of 1 drop to 1 ounce, is a still more efficient preservative, and is especially useful for microscopic structures; but it will interfere with Obermayer's test for indican, and if larger amounts be used, it may give reactions for sugar and albumin, and is likely to cause a precipitate which greatly interferes with the microscopic examination. Thymol, toluol, and chloroform are likewise much used. The use of thymol is very convenient. A small lump, floating on the surface, will preserve a bottle of urine for several days, but enough may dissolve to simulate the albumin reaction. The chief objection to toluol is the fact that it floats upon the surface, and the urine must be pipeted from beneath it. It is, however, the best preservative for the chemical constituents, particularly acetone and diacetic acid. Chloroform is probably the least satisfactory. It reduces Fehling's solution; and it settles to the bottom in the form of globules which it is impossible to avoid when removing the sediment for microscopic examination. One of these preservatives may be placed in the vessel when collection of the twenty-four-hour sample is begun. Whenever possible the urine should be kept on ice.

Normal and **abnormal pigments,** which interfere with certain of the tests, can be removed by filtering the urine through animal charcoal, or precipitating with a solution of normal acetate of lead (sugar of lead), or with powdered lead acetate in substance, and filtering.

Certain cloudy urines cannot be **clarified** by ordinary filtration through paper, particularly when the cloudiness is due to bacteria.

Such urines can usually be rendered comparatively clear by adding a small amount of purified talc or infusorial earth, shaking well, and filtering.

A suspected fluid can be **identified as urine** by detecting any considerable quantity of urea in it (p. 121). Traces of urea may, however, be met with in ovarian cyst fluid, while urine from very old cases of hydronephrosis may contain little or none.

The **frequency of micturition** is often suggestive in diagnosis. Whether it is unduly frequent can best be ascertained by asking the patient whether he has to get up at night to urinate. Increased frequency may be due to restlessness; to increased quantity of urine; to irritability of the bladder, usually an evidence of cystitis; to obstruction ("retention with overflow"); or to paralysis of the sphincter.

The points to be covered in a **routine examination** will vary with circumstances. The following are suggested for office work and for the small hospital: Quantity, if twenty-four-hour amount is available; color and transparency; reaction; specific gravity; qualitative tests for albumin, sugar, indican, and acetone bodies; and a careful microscopic examination of the sediment.

Clinical examination of the urine may conveniently be considered under five heads: I. General characteristics. II. Functional tests. III. Chemical examination. IV. Microscopic examination. V. The urine in disease.

I. GENERAL CHARACTERISTICS

1. Quantity.—The quantity passed in twenty-four hours varies greatly with the amount of liquids ingested, perspiration, etc. The normal average may be taken as 1,200 to 1,500 c.c., or 40 to 50 ounces for an adult in this country. German writers give higher figures. For children the amount is somewhat greater in proportion to body weight.

The quantity is increased (polyuria) during absorption of large serous effusions and in many nervous conditions. It is usually much increased in chronic interstitial nephritis, diabetes insipidus, and diabetes mellitus. In these conditions a permanent increase in amount of urine is fairly constant—a fact of much value in diagnosis. In diabetes mellitus the urine is usually 2 to 5 liters, and may, though rarely, reach the enormous amount of 28 liters.

The quantity is decreased (oliguria) in severe diarrhea; in fevers; in all conditions which interfere with circulation in the kidney, as poorly compensated heart disease; in the parenchymatous forms of nephritis; and during accumulation of fluid in the serous cavities. In uremia the urine is usually very greatly decreased and may be entirely suppressed (anuria).

Ordinarily, more urine is voided during the day than during the night, the normal ratio being about 4 to 1 or 3 to 1. *Nocturnal polyuria*, in which the night urine (8 A. M. to 8 P. M.) is increased and may equal or exceed that passed during the day, is of value as a sign of early functional derangement of the kidneys provided no water is taken after the evening meal (Mosenthal's Test, p. 101).

2. Color.—This varies considerably in health, and depends largely upon the quantity of urine voided, dilute urines being pale and concentrated urines highly colored. The usual color is yellow or reddish yellow, due to the presence of several pigments, chiefly urochrome, which is yellow. Traces of hematoporphyrin, uro-erythrin, and urobilin are frequent. Uro-erythrin is chiefly responsible for the deep reddish tinge of urine in acute fevers. Urobilin and hematoporphyrin have clinical significance and are discussed later (pp. 158 and 162). Acid urine is generally darker than is alkaline. For the sake of uniformity in recording the color, Vogel's scale is widely used, the urine being filtered and examined by transmitted light in a glass 3 or 4 inches in diameter. This scale uses nine colors, pale yellow, light yellow, yellow, reddish yellow, yellowish red, red, brownish red, reddish brown, and brownish black.

Color is sometimes greatly changed by abnormal pigments. Blood-pigment gives a red or brown, smoky color. Urine containing bile is yellowish or brown, with a yellow foam when shaken. It may assume a greenish hue after standing, owing to oxidation of bilirubin into biliverdin. Ingestion of small amounts of methylene-blue gives a pale green; large amounts give a marked greenish blue. Santonin produces a yellow; rhubarb, senna, cascara, and some other cathartics, a brown color; these change to red upon addition of an alkali, and if the urine be alkaline when voided may cause suspicion of hematuria. A bright pink or red color appearing when the urine is alkalinized may be due to phenolphthalein taken as a

laxative. Thymol gives a yellowish green. Following poisoning from phenol and related drugs the urine may have a normal color when voided, but becomes olive green to brownish black upon standing. Urine which contains melanin, as sometimes in melanotic tumors, and very rarely in wasting diseases, also becomes brown or black upon long standing. A similar darkening upon exposure to the air occurs in alkaptonuria (p. 162). A milky color may be due to presence of chyle, or milk may have been added by a malingering patient.

A pale greenish urine with high specific gravity strongly suggests diabetes mellitus.

3. Transparency.—Freshly passed normal urine is clear. Upon standing, a faint cloud of mucus, leukocytes, and epithelial cells settles to the bottom—the so-called "nubecula." This is more abundant in women, owing to vaginal cells and mucus. In urines of high specific gravity it may float near the middle of the fluid.

Abnormal cloudiness is usually due to presence of phosphates, urates, pus, blood, or bacteria. Epithelial cells and tube-casts are rarely present in sufficient number to produce more than a slight cloudiness, although they may add to turbidity due to other causes. There are on record a very few cases in which cloudiness was caused by spontaneous precipitation of albumin, but, in general, albumin does not affect the transparency of the urine. The presence of albumin does, however, cause marked foaming when the urine is shaken.

Amorphous phosphates are precipitated in neutral or alkaline urine. They form a white cloud and sediment, which disappear upon addition of an acid.

Amorphous urates are precipitated only in acid urine. They form a white or pink cloud and sediment ("brick-dust deposit"), which disappear upon heating.

Pus resembles amorphous phosphates to the naked eye. Its nature is easily recognized with the microscope, or by adding a strong solution of caustic soda to the sediment, which is thereby transformed into a gelatinous mass (Donné's test).

Blood gives a reddish or brown, smoky color, and may be recognized with the microscope or by tests for hemoglobin.

Bacteria, when present in great numbers, give a uniform cloud,

which cannot be removed by ordinary filtration. They are detected with the microscope.

The cloudiness of decomposing urine is due mainly to precipitation of phosphates and multiplication of bacteria.

4. Odor.—The characteristic aromatic odor has generally been attributed to volatile acids. A substance, called "urinod," has also been held responsible. The odor is most marked in concentrated urines. During decomposition the odor becomes ammoniacal. A fruity odor is sometimes noted in diabetes, due probably to acetone. Urine which contains cystin may develop an odor of sulphureted hydrogen during decomposition.

Various articles of diet and drugs impart peculiar odors. Notable among these are asparagus, which gives a characteristic offensive odor, and turpentine, which imparts an odor somewhat suggesting that of violets.

5. Reaction.—Normally, the mixed twenty-four-hour urine is slightly acid in reaction. The acidity sometimes increases for a time after the urine is voided, the so-called "acid fermentation." The acidity was formerly held to be due wholly to acid phosphates, but Folin has shown that the acidity of a clear urine is ordinarily greater than the acidity of all the phosphates, the excess being due to free organic acids. Individual samples may be slightly alkaline, especially after a full meal; or they may be amphoteric, turning red litmus-paper blue and blue paper red, owing to presence of both alkaline and acid phosphates. The reaction is ordinarily determined by means of litmus-paper, which, however, is worthless unless of good quality. That put up in vials by Squibb can be recommended.

The hydrogen-ion concentration or true reaction of the urine is ordinarily about *p*H 6, with the normal range of 4.8 to 7.5. Simple outfits for the determination of hydrogen-ion concentration colorimetrically can be purchased from the chemical supply houses.

Acidity is increased after administration of certain drugs, by excess of protein in the diet, in acidosis, and whenever the urine is concentrated from any cause, as in fevers. A strongly acid urine may cause frequent micturition because of its irritation. This is often an important factor in the troublesome enuresis of children.

Quantitative estimation of acidity of urine is not of much clinical value. When, however, it is desired to make it, the method of Folin will be found satisfactory. In every case the sample must be from the mixed twenty-four-hour urine and as fresh as possible.

Folin's Method.—Into a small flask measure 25 c.c. of the urine, and add 1 or 2 drops 0.5 per cent. alcoholic solution of phenolphthalein and 15 or 20 gm. of neutral potassium oxalate. Shake for a minute, and immediately titrate with decinormal sodium hydroxid, shaking meanwhile, until the first permanent pink appears. Read off from the buret the amount of decinormal sodium hydroxid solution added, and calculate the number of cubic centimeters which would be required for the entire twenty-four hours' urine. Most estimations run between 25 and 40 c.c. of decinormal solution for 100 c.c. of urine. Folin places the normal acidity for the twenty-four-hour specimen at 554 to 669 c.c. of decinormal solution, but most other authors give lower figures. Much depends on the diet.

The urine always becomes alkaline upon long standing, owing to decomposition of urea with formation of ammonia. Marked alkalinity of the freshly voided urine usually indicates such "ammoniacal decomposition" in the bladder, which is the rule in neglected chronic cystitis, especially that due to paralysis or obstruction. This form of alkalinity is known as *volatile alkalinity*, and can be recognized by the odor or by the fact that litmus-paper turned blue by the urine again becomes red upon gentle heating, or that the paper will turn blue when held in the steam over the boiling urine. Such accumulation of free ammonia, derived from breaking down of urea after the urine is secreted and leading to volatile alkalinity of the urine, is frequently confused in the student's mind with increased elimination of ammonium salts in acidosis, which is associated with increased acidity. A second form of alkalinity, *fixed alkalinity*, is due to alkaline salts, and is often observed during frequent vomiting, after the crisis of pneumonia, in various forms of anemia, during digestion of full meals ("alkaline tide"), after abundant eating of fruits, and after administration of certain drugs, especially salts of vegetable acids. In some cases, as a result of the alkalinity, there is regularly a white deposit of amorphous phosphates, and the condition has been called "phosphatic diabetes."

With normal individuals, administration of 3 to 5 gm. of sodium bicarbonate by mouth will cause the urine to become alkaline. In conditions of acidosis, upon the other hand, very much larger amounts of bicarbonate may be given without bringing about this change. It appears that a large proportion of the carbonate is retained in the body to fortify the depleted alkali reserve of the blood and tissues. Only after the reserve is restored does the carbonate pass into the urine and change its reaction. This fact forms the basis for the "bicarbonate tolerance test" of Sellards,[1] which is a simple and useful test for acidosis. Unlike the determination of ammonium salts in urine or detection of acetone, it is applicable in all forms of acidosis. Moreover, it furnishes a reasonably accurate index of the degree of acidosis, although Palmer and Van Slyke have shown that, judged by the CO_2-combining power of blood-plasma, it generally indicates a somewhat greater degree of acidosis than really exists.

The test consists in giving the patient 5 gm. of sodium bicarbonate, dissolved in a little water, by mouth, every two or three hours until the urine, voided before each dose, becomes neutral or faintly alkaline to litmus paper. The urine is thoroughly boiled before testing.

Tolerance of 20 to 30 gm. of bicarbonate indicates a moderate grade of acidosis which usually produces no clinical symptoms. Tolerance of 40 to 50 gm. is noted in more marked grades which still do not lead to symptoms beyond dyspnea upon excretion. When the tolerance reaches 75 to 100 gm. there may be very definite and serious clinical symptoms. In extreme cases the tolerance may reach 150 gm.

Since, however, large amounts of bicarbonate are not well borne by the stomach it is well, in severe acidosis, not to push the administration until the urine becomes alkaline, but to discontinue as soon as the existence of marked acidosis is established.

6. Specific Gravity.—In a general way this varies inversely with the quantity of urine. The normal average is about 1.017 to 1.020. Samples of urine taken at random may go far above or below these figures. That first voided in the morning is generally most concentrated. Normal kidneys should dilute the urine to a specific gravity of 1.003 or less following ingestion of 1500 c.c. of water upon an empty stomach in the morning, and should be able to concentrate the urine to about 1.030 when the patient is upon a diet of solid food without liquids for a day. Inability to dilute or

[1] For further details and directions for sterilization and intravenous use of the bicarbonate, see Sellards, A. W.: The Principles of Acidosis and Clinical Methods for its Study, Harvard University Press, 1919.

concentrate to this degree is evidence of defective renal function (Concentration and Dilution Test of Volhard and Fahr, page 103). Variations in specific gravity of specimens, taken at stated intervals during the day when the patient is upon a standard diet with no intake between meals, are also of value in detecting functional incapacity of the kidneys and are discussed on page 101.

Pathologically, the specific gravity of the mixed twenty-four-hour urine may vary from 1.001 to 1.060. It is low in chronic interstitial nephritis, diabetes insipidus, and many functional nervous disorders. It is high in fevers and in parenchymatous disease of

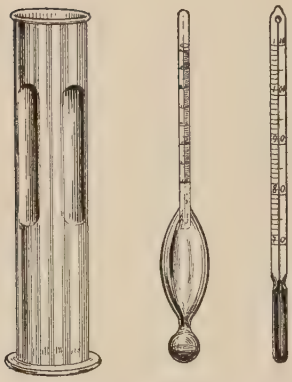


FIG. 33.—Squibb's urinometer with thermometer and cylinder.

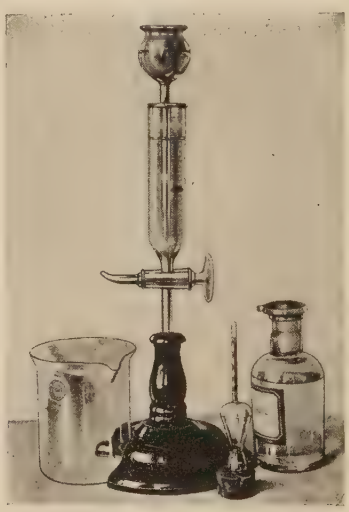


Fig. 34.—Exton's immiscible balance.

the kidney. In any form of nephritis a sudden fall without a corresponding increase in quantity of urine may foretell approaching uremia. It is highest in diabetes mellitus. A high specific gravity when the urine is not highly colored, or when the quantity is above the normal, should lead one to suspect this disease. A normal or low specific gravity, even below 1.005, does not, however, exclude it.

The specific gravity is most conveniently estimated by means of the urinometer (Fig. 33). Squibb's urinometer is adjusted to give accurate readings at 22.5° C.; the Tycos instrument, at 25° C. If the urine be brought to about the right temperature, a correction

for temperature will seldom be necessary in clinical work. For accuracy, however, it is necessary to add 0.001 to the urinometer reading for each 3° C. above the temperature for which the urinometer is standardized, and to subtract 0.001 for each 3° C. below that point. Care should be taken that the urinometer does not touch the side of the tube, and that air-bubbles are removed from the surface of the urine. Bubbles are easily removed with a strip of filter-paper. With most instruments the reading is taken from the bottom of the meniscus. A long scale on the stem is desirable because of the greater ease of accurate reading. Many of the urinometers on the market are too small to be of any real value.

The specific gravity of extremely small quantities of fluid can be ascertained by the following method which has been much used for the specific gravity of the blood: Two fluids, such as benzol and chloroform, which differ widely in specific gravity and which readily mix with each other, but do not mix with the fluid to be tested, are mixed in a cylinder. A drop of the fluid to be tested is then placed in the mixture, and the specific gravity of the mixture is adjusted by adding the lighter or the heavier of the fluids until the drop remains suspended near the middle. The mixture then has the same specific gravity as the fluid which is being tested, and this is readily ascertained with an accurate hydrometer. The following precautions must be observed: (*a*) The two fluids must be well mixed. (*b*) The drop of fluid to be tested must be of medium size, it must not contain an air-bubble, it must not cling to the side of the cylinder, and it must not remain long in the fluid. (*c*) After the specific gravity has been adjusted as accurately as possible with the first drop, it should be verified with a fresh drop. (*d*) The hydrometer must be standardized for the chloroform-benzol mixture or other fluid used in the test. Make a mixture such that a drop of distilled water will remain suspended in it (that is, with specific gravity of 1.000) and correct the hydrometer by this.

Exton's "immiscible balance" for the determination by the above method of the specific gravity of small quantities of urine, blood, or most aqueous solutions is especially satisfactory (Fig. 34). He uses a mixture of carbon tetrachlorid and a petroleum product sold as "varnolene," "stanisol," and possibly under other trade names.

7. Total Solids.—The total amount of solids which pass through the kidneys in twenty-four hours is about 60 gm., or 950 grains for a man of 150 pounds. The principal factors which

influence this amount are body weight (except with excessive fat), diet, exercise, age, the activity of metabolism, and the ability of the kidneys to excrete. Simple estimations of the total solids, therefore, may under uniform conditions of diet and exercise furnish an important clue to the functional efficiency of the kidneys. After about the forty-fifth year the solids become gradually less; after the seventy-fifth the normal amount is about one-half that given above.

The total solids can be estimated roughly, but accurately enough for most clinical purposes, by multiplying the last two figures of the specific gravity of the mixed twenty-four-hour urine by the number of ounces voided and to the product adding one-tenth of itself. This gives the amount in grains. If, for example, the twenty-four-hour quantity is 3 pints or 48 ounces, and the specific gravity is 1.018, the total solids would approximate 950 grains, as follows:

$$48 \times 18 = 864; 864 + 86.4 = 950.4.$$

This method is especially convenient for the practitioner, because patients nearly always report the amount of urine in pints and ounces, and it avoids the necessity of converting into the metric system. Häser's method, which uses the metric system, is more widely used, but is less convenient. The last two figures of the specific gravity are multiplied by 2.33. The product is then multiplied by the number of cubic centimeters voided in twenty-four hours and divided by 1000. This gives the total solids in grams.

II. TESTS OF KIDNEY FUNCTION

With the growing appreciation that it is usually the altered function of a diseased organ that measures its menace to the patient's health and that the functional disturbance may be the first definite indicator of anatomic changes, much thought has been given within recent years to the development of methods which seek to ascertain whether the kidneys are capable of performing the work which the activities of normal life demand of them, and to measure the degree of insufficiency when they fail in this respect. Such methods afford invaluable aid in the study of kidney disease and of certain extrarenal conditions which interfere with kidney function, and they furnish a useful guide to prognosis and treatment as well.

It must be borne in mind, however, that the tests measure

function only, not the extent of anatomic change in the kidney; and these do not necessarily go hand in hand even when definite organic disease exists. As is well known, kidney function may be markedly depressed by conditions which are primarily extrarenal, such as heart disease with poor compensation, severe general anemia, hypertrophied prostate, and cystitis; while, upon the other hand, some of the tests may show exalted functional activity in early stages of kidney disease. Moreover, since the kidney's function is multiple, some phases of its activities may be markedly interfered with while others remain normal; and the different tests, which do not necessarily measure the same function, may sometimes give apparently inconsistent results in a given case. For this reason it is highly desirable that two or more of the tests be employed in every case.

No less than fifty tests of renal function have been proposed, but only a few have received wide acceptance. Even the simple calculation of total solids, described upon a previous page, has much value in this field. Of the more important functional tests the following are perhaps the most useful for clinical work: (*a*) Phenolsulphonephthalein test; (*b*) Mosenthal's test-meal for renal function; (*c*) estimation of nitrogen retention; (*d*) Ambard's coefficient. Of these, the first two are so simple as to be easily carried out in private practice. The other two demand special training in the technic of blood chemistry.

The limitations of these four functional tests and their application to the study of nephritis and other conditions are discussed at some length in the section upon Nephritis. The following summary of their comparative value and special fields of usefulness seems to be warranted:

1. The test-meal for renal function affords, particularly in the character of the night urine, the most sensitive indicator of defective kidney function, and is especially useful for early diagnosis of chronic nephritis. It may, indeed, indicate grades of functional disturbance which are so slight that they may be disregarded.

2. The phenolsulphonephthalein test and Ambard's coefficient run closely parallel, and are most useful in mild, moderate, and severe grades of insufficiency. The latter is thought by some, but by no means all, of those who have used it to be slightly more reliable; but the former is certainly preferable for practical work be-

cause of its simplicity, and may be accepted as our most useful test for clinical purposes.

3. The estimation of nitrogen-retention by determination of blood urea finds its chief usefulness in the latter stages of kidney disease, where it is the most reliable prognostic sign. It is also useful in distinguishing passive congestion due to heart disease. Owing to the wide range of normal variations, it fails to yield unmistakable evidence as to the condition of the kidney in slight and moderate grades of insufficiency.

4. Owing to the remarkable reserve power of the kidneys, whereby one kidney can, in the absence of any unusual strain, carry on the work of the two, *bilateral* disturbance may be inferred when these tests indicate a notable degree of renal insufficiency, and particularly when nitrogen is retained. When there is a question of *unilateral* kidney disease a special method must be employed; and, of the tests described here, only the phenolsulphonephthalein test is applicable. Its modification for this purpose is described on page 100.

The table on page 97 shows the values to be expected of the different tests in various grades of kidney insufficiency.

1. Phenolsulphonephthalein Test.—This test, which was offered by Rowntree and Geraghty in 1910, consists in the intramuscular or intravenous injection of a solution of phenolsulphonephthalein, a drug which is eliminated only by the kidneys, and whose amount in the urine is easily estimated by colorimetric methods. The time of its first appearance in the urine and the quantity eliminated within a definite period are taken as a measure of the functional capacity of the kidneys. The test is harmless, extremely simple, and for general purposes the most satisfactory of the functional tests. Other substances—methylene-blue, indigocarmin, etc. —have been used in a similar manner, as well as urea and creatinin by mouth, but have not met with general approval.

Technic.—The original procedure, in which the patient was catheterized when the drug was injected and the catheter was left in place until the drug was detected in the urine, is now seldom followed. The catheter is still used if there be obstruction to the outflow of urine, but ordinarily it is dispensed with, and the procedure is as follows:

1. Give the patient 300 to 400 c.c. (about 2 glasses) of water to promote secretion of urine.

SCALE OF DEGREES OF RENAL INSUFFICIENCY AND COMPARISON OF VALUES YIELDED BY THE MORE IMPORTANT TESTS OF KIDNEY FUNCTION

State of renal function.	Phenol-sulphone-phthalein excretion, per cent.	Non-protein nitrogen of the blood mgm. per 100 c.c.	Urea nitrogen of the blood mgm. per 100 c.c.	Ambard's coefficient of urea excretion.	Test-meal for renal function:					
					Night urine.		Variations in specific gravity of two-hour specimens when the highest specific gravity is			
					Volume.	Specific gravity.	1018.	1017 to 1013.	1014 and 1013.	1012 or less.
Normal.....	60 or more	30 or less	15 or less	0.090 or less	500 c.c. or less	1018 or more	9 or more			
Slight impairment..	59 to 40	31 to 45	16 to 27	0.091 to 0.115	500 c.c. to 750 c.c.	1016 and 1017	8 to 5	6 or more		
Moderate " ..	39 to 25	46 to 65	28 to 44	0.116 to 0.220	750 c.c. or more	1015 or less	4 or less	5 and 4	6 or more	
Marked " ..	24 to 11	66 to 90	45 to 64	0.221 to 0.350	750 c.c. or more	1015 or less	4 or less	3 or less	4 and 5	6 or more
Maximal " ..	10 to 0	91 or more	65 or more	0.351 or more	750 c.c. or more	1015 or less	4 or less	3 or less	3 or less	5 or less

Slightly modified from Mosenthal, H. O., and Lewis, D. S., Jour. Amer. Med. Assoc., vol. 67, p. 933, September 23, 1916.

2. Twenty minutes afterward have him empty his bladder and discard the urine. Then with a hypodermic syringe inject exactly 1 c.c. of the sterile phenolsulphonephthalein solution[1] intramuscularly, preferably into the deltoid, gluteal, or lumbar muscles. If there be general edema, hindering absorption, it should be injected intravenously.

3. In exactly one hour and ten minutes from the time of the injection have the patient empty his bladder and save all the urine. The ten-minute period represents the usual time which elapses between the injection and the first appearance of the dye in the urine.

4. In two hours and ten minutes after the injection have the patient empty his bladder again, and save all the urine in a separate container. He should be under observation during the two-hour period, else it is difficult to make sure that he carries out his instructions exactly.

5. Estimate the output of phenolsulphonephthalein in each of the two portions of urine separately as described below.

Estimation of Output.—Record the volume of each of the one-hour portions of urine. If either is very low—less than 40 c.c.—results are not dependable. To each of the two portions add sufficient sodium hydroxid solution to bring out the maximum purplish-red color, and estimate the amount of the drug contained in each by comparing the color with that of an alkalinized standard solution. The result is recorded in terms of the percentage of the amount injected.

In detail, this is done as follows:

1. In a graduate or volumetric flask dilute each of the one-hour specimens of urine with water to about 800 c.c., add about 5 c.c. of 10 per cent. sodium hydroxid solution or enough to bring out the maximum purplish-red color, and bring each to 1000 c.c. with water. Mix well.

2. Add 1 c.c. of the phenolsulphonephthalein solution to about 800 c.c. of water, alkalinize with sodium hydroxid, and dilute to 1000 c.c. Since this contains the same amount of the drug as was injected, it may be rated as a 100 per cent. standard-color solution. As a rule no more than 100 c.c. of the standard solution will be needed, and there usually will be enough of the solution left in the original ampule to make this amount.

3. Filter each of the diluted and alkalinized specimens of urine and compare with the 100 per cent. standard solution in any good colorimeter. The use of colorimeters is described on page 107. The simple and inexpensive Denison laboratory instrument is especially useful for this

[1] This solution may be obtained of any druggist. It is sold in 1-c.c. ampules, sterilized ready for use; but it should be noted that these ampules contain somewhat more than 1 c.c., hence one should not inject the entire contents.

purpose. Results with this and the Duboscq type are most dependable when the unknown solution and the standard have nearly the same depth of color. It is therefore well to use a 50 per cent. standard solution made by diluting the 100 per cent. standard above recommended with an equal amount of water and mixing well. The Dunning colorimeter (Fig. 35) consists of thirteen sealed ampules containing standard color solutions of different percentages, an open ampule in which the unknown specimen is placed, and a small box in which the specimen is compared with the standards. It is very satisfactory for office work because the physician need not make his own standard solution. We have found the colors to remain for over a year with very little fading when kept in the dark.

4. In the absence of a colorimeter one can easily make up a series of standards, representing 10, 20, 30 per cent., and so forth, by diluting the 100 per cent. standard. The unknown is then matched against these

FIG. 35.—Dunning's colorimeter for the phenolsulphonephthalein test of kidney function.

in test-tubes of the same diameter. The standard solution which matches the unknown indicates the percentage of excretion. If, for example, the first hour's specimen is matched by the 40 per cent. standard, and the second hour's specimen by the 20 per cent. standard, then the phenolsulphonephthalein output for the two periods is 40 and 20 per cent., respectively, and for the two hours is 60 per cent.

In order to equalize the slight difference in color due to a highly colored urine, the standard color may be viewed through a faintly yellow-tinted piece of glass, or an amount of normal urine sufficient to give the desired shade may be included in the standard solution. For those who do much work it is convenient to add a few drops of a solution of some yellow dye, such as Echtgelb G or tropeolin OO.

When it is necessary to defer the color comparison for hours or days the urine must be kept acid, as the color fades in alkaline solution. Phosphoric acid is best for this purpose. A standard solution made without urine will remain for some months with only slight fading.

Under normal conditions the drug first appears in the urine in five to eleven minutes after the injection. Within the first hour after its appearance 40 to 50 per cent. is eliminated; in the two hours, 60 to 75 per cent. Pathologically, the excretion may be reduced to a trace or even, in extreme cases, to none at all in the two hours. The values to be expected in various grades of kidney insufficiency are indicated in the table on page 97. The test should not be applied when the patient is taking saline cathartics, as they tend to delay excretion.

Time has proved the great usefulness of this test in every-day practice. It will sometimes reveal a serious degree of renal failure when other urinary findings are practically normal, but it must be remembered that it is a test of functional capacity only, not a measure of the extent of anatomic changes in the kidney, and that no one of the functional tests covers the entire range of the kidney's activities. The test is extremely valuable in diagnosis and prognosis of nephritis, and in this connection is discussed more fully on page 210. It is most useful in chronic interstitial nephritis where the phenolsulphonephthalein output runs fairly parallel with the course of the disease. In very early nephritis there may be excessive elimination, owing probably to irritation and overaction of undamaged portions of the kidney. In such cases this test should be supplemented by Mosenthal's test-meal for renal function. In acute nephritis the output does not always agree with the clinical and pathologic picture. Particularly is this true in the acute glomerulonephritis of scarlet fever, where the excretion percentage may sometimes be fully up to the normal. Apparently the test speaks less definitely concerning glomerular changes than tubular.

The output is low in heart disease with chronic passive congestion of the kidneys and rises when the congestion is relieved by improved heart action. Low values are also often found in prostatic hypertrophy and in cystitis with retention of urine.

Application of the Test to the Two Kidneys Separately.—This requires catheterization of the ureters, the patient being given two glasses of water about one-half hour beforehand and the phenolsulphonephthalein solution being injected intravenously immediately after the catheters are in place.

The urine is received from the catheters directly into two test-tubes which contain a few drops of 10 per cent. sodium hydroxid solution.

The time of first appearance of the dye in the urine, indicated by the appearance of a red color in the tubes, is noted, and the urine is collected for one-half or one hour thereafter in fifteen-minute or half-hour periods. The output of the drug in each of the specimens is then determined as already described.

Under normal conditions the dye first appears in the urine in three to five minutes after intravenous injection, although it may sometimes be delayed for one or both kidneys as a result of reflex inhibition due to the presence of the catheters. The total output for each kidney is more important than the time of appearance. This for the two kidneys together is about 35 to 45 per cent. in fifteen minutes, 50 to 60 per cent. in the first half-hour, and 65 to 80 per cent. in the first hour. When one kidney is defective the appearance of the drug in the corresponding urine is greatly delayed and the total elimination from this kidney is reduced. In such cases the other kidney may compensate to greater or less degree by increased output. When the catheters are removed care should be taken to ascertain whether any urine has leaked past them into the bladder. This accident would confuse results.

2. Test-meal for Renal Function.—Following the work of Hedinger and Schlayer and others, Mosenthal has recently placed upon a practical footing a valuable test of renal function which is based upon the characteristics of the urine—chiefly volume of the night urine and variations in specific gravity of two-hour specimens taken during the day—when the patient is upon a prescribed diet.

When the kidneys are healthy, the urine which they excrete at different periods within the twenty-four hours varies markedly in volume and specific gravity. These variations represent the ready response of normal kidneys to the varying demands for the elimination of water or solids imposed by the necessity of maintaining the concentration of the body fluids at a constant level in spite of the periodic intake of food and water. Diseased kidneys, upon the other hand, lose this adaptive power to greater or less degree, and the limits within which they can vary their activities become greatly narrowed. The urine which they excrete consequently remains of almost uniform concentration from hour to hour. Numerous modifications of this test are now in use, but only the original method need be described here.

Method in Detail.—Mosenthal originally prescribed a definite, weighed and measured test diet, but this is no longer thought essential.

1. Upon the day of the test, and preferably also the day before, place the patient upon a full diet, such as the following: Breakfast of fruit, cereal, bread, butter, and tea, coffee, cocoa, or water at 8 A. M.; dinner of soup, meat, vegetables, bread, butter, dessert, and tea, coffee, or water at noon; and supper of eggs, bread, butter, fruit, and tea or water at 5 P. M. Much latitude is allowable in the choice of foods, and in many cases the ordinary diet to which the patient is accustomed may be used. At least a pint of fluid—tea, coffee, water, etc.—must be taken at each meal; and no food or liquid of any sort may be taken outside of these meals until after 8 o'clock the following morning.

2. Instruct the patient to empty his bladder immediately before breakfast. Collect specimens of urine at 10 A. M.; 12 noon; 2 P. M.; 4 P. M.; 6 P. M.; 8 P. M.; and, finally, at 8 o'clock the following morning. It is essential that the intervals be exact and that the bladder be completely emptied each time. Should the hour for the meals be changed, the times of collecting the samples of urine should be changed accordingly. The last of the two-hour specimens must not be collected less than three hours after the beginning of the evening meal.

3. Measure the night urine (8 P. M. to 8 A. M.) and take its specific gravity with an accurate urinometer.

4. Measure the six two-hour specimens and take their specific gravity, first making sure that they are all at the same temperature, since misleading figures may be obtained if some have been kept on ice and some at room temperature.

Normal Values.—In health the urinary response is as follows:

1. The night urine will be much less than the total day urine. It is usually 250 to 350 c.c., and will seldom exceed 400 to 500 c.c.; 750 c.c. is the maximum. Its specific gravity will usually be 1.018 or above.

2. The highest specific gravity recorded for the two-hour day specimens will exceed 1.018, while the difference between the highest and the lowest will not be less than 8 or 9 points. If, for example, the most concentrated specimen has a specific gravity of 1.020, the most dilute will be 1.011 or less.

Indications of Impaired Renal Function.—One or more of the following changes may be noted:

1. *Nocturnal Polyuria.*—The volume of the night urine exceeds 750 c.c. This is usually one of the first and most definite evidences of impaired kidney function. A volume between 500 and 750 c.c. is suspicious, and usually indicates impairment. As H. W. Jones has pointed out, a determination of the ratio between day and night urine (p. 87) is especially helpful in interpreting these borderline figures.

2. *Low maximal specific gravity* of day urine, the highest of the two-hour specimens falling below 1.018.

3. *Fixation of specific gravity*, that is, lessened variations in the specific gravities of the two-hour specimens. This is a very important sign of renal insufficiency. In marked cases the difference between the highest and lowest specific gravities may be only one or two points. As a rule, the level at which the specific gravity is fixed becomes lower as the functional impairment increases and the kidneys lose their ability to concentrate urine.

Fixation of specific gravity at a high level, near 1.018 or 1.020, may occur in acute nephritis, chronic parenchymatous nephritis, and passive congestion of the kidney, but is not necessarily an indication of disease, since it may occur when the patient has been taking insufficient water, or when there has been excessive loss of water through perspiration. Absorption and elimination of edema at the time of the test, leading to fixation of specific gravity at a low level, may also confuse the results.

3. Concentration and Dilution Tests.—Volhard and Fahr have suggested two very simple tests for kidney function, one measuring concentrating power, and the other, diluting power. The concentration should be done first and twenty-four hours allowed to elapse before beginning the dilution test.

Concentration Test.—Allow no fluids from the evening before the test until the test is finished, and no food between meals.

8 A. M., Breakfast: Dry cereal with sugar, syrup, or honey; no milk; one egg; toast or bread with butter.

12 Noon, Dinner: Roast beef, steak, or chops; potatoes, boiled, baked, or riced; bread and butter; jam.

5 P. M., Supper: Two eggs; bread and butter; jam.

8 A. M. of same day: Empty bladder. Collect urine in separate containers every three hours thereafter until night, that is, at 11 A. M., 2 P. M., 5 P. M., 8 P. M.; and collect all urine from 8 P. M. to 8 A. M. next morning in one container.

Note the quantity and specific gravity of each three-hour sample and of the twelve-hour sample and plot as a curve. Normally, the specific gravity of at least one sample should be 1.030, or at least 1.025.

Dilution Test.—This is also known as the "water test."

Omit breakfast. For dinner and supper give the routine nephritic diet or the diet to which the patient has been accustomed. Permit one glass of water after supper.

8 A. M.: Empty bladder and give 1500 c.c. water.

Collect urine in separate containers at 8.30, 9.00, 9.30, 10.00, 10.30, 11.00, 11.30 A. M., and 12.00 noon, eight specimens in all. Collect all the urine from 12.00 noon to 8.00 next morning in one container.

Note the quantity and specific gravity of each sample and plot as a curve. Normally, the total quantity voided should be 80 to 120 per cent. of the intake (1200 to 1800 c.c.). The specific gravity of at least one sample should be as low as 1.003.

The two tests given above, particularly the first, show decreased renal efficiency often before it can be demonstrated with other standard renal tests. They are so simple that they can be done in general practice, and without any elaborate equipment.

4. Nitrogen Retention as Evidence of Kidney Insufficiency.—The nitrogen-containing substances of the blood may conveniently be divided into two classes: (1) proteins, which are essential and permanent constituents of the blood; and (2) various non-protein nitrogenous substances whose transportation is one of the chief functions of the blood; partly transformed foodstuffs, which, unless utilized by the tissues, circulate until excreted by the kidneys; partly waste products of metabolism which also must be excreted. It is the non-protein group which concerns us here. Although the quantity to be eliminated varies greatly from hour to hour, depending upon intake of food or exercise, yet through the adaptability of the kidney the concentration in the blood is kept at a fairly constant level, with a rise for only a short period following meals. When the kidneys are damaged and their capacity to excrete thereby lessened, these substances tend to accumulate in the blood. To this condition, in which chemical analysis of the blood shows abnormally large and sometimes enormous amounts of non-protein nitrogen, the name "nitrogen retention" is applied; and, other things being equal, the extent to which it occurs may be taken as an index of the degree of disability of the kidneys. Among the retained substances urea holds first place, amounting to about one-half of the whole. Others, which need not be considered here, are uric acid and creatinin.

The following figures represent the values obtained in health under ordinary conditions of diet and exercise:

Total non-protein nitrogen	25 to 30	mg.	per 100 c.c.	of blood
Urea-nitrogen[1]	12 to 15	"	"	"
Uric acid	2 to 3	"	"	"
Creatinin	1 to 2	"	"	"

In health and disease the concentration of these substances in the blood is the resultant of three variable factors: Intake of nitrogenous food, activity of metabolism, and excretory ability of the kidneys. To these must be added in certain cases the formation of extensive edema or large exudates which "lock up" a considerable amount of urea.

Owing to the impracticability of fully controlling the diet and accurately measuring the balance of metabolism in clinical work and to the remarkable reserve power of the kidneys, estimations of the non-protein nitrogenous substances are of comparatively small value for detecting and measuring slight grades of kidney disability. In more advanced grades, upon the other hand, when nitrogen retention is sufficiently marked to be unquestioned, these estimations probably constitute the most helpful of all functional tests, particularly as a guide to prognosis and treatment and as an aid in the differential diagnosis of renal and cardiac disorders, in the latter of which nitrogen does not accumulate in the blood. The figures which may be expected with this and other functional tests in various grades of kidney disability are given in the table on page 97.

To estimate the degree of nitrogen retention some workers determine the total non-protein nitrogen of the blood, others rely upon urea-nitrogen alone. It apparently makes little difference which of the two is determined, since the results run closely parallel under practically all circumstances. Estimation of urea is much simpler and is therefore given preference here.

Further discussion of nitrogen retention in relation to kidney disease, particularly the significance of blood uric acid and creatinin, will be found on page 211. Methods of estimating nitrogen retention are described in the section upon Chemical Examination of the Blood, page 332.

5. Ambard's Coefficient and McLean's Index.—As has already been stated, the concentration of urea in the blood cannot be unreservedly relied upon as a measure of slight insufficiency of the

[1] The distinction between *urea* and *urea-nitrogen* must be kept in mind. One gram of urea-nitrogen corresponds to 2.14 gm. of urea. Blood urea is usually recorded in terms of urea-nitrogen.

kidneys, owing to the rather wide variations which are possible as a result of extrarenal causes. From the studies of Ambard and others, it appears that a much better measure of the kidney's ability to excrete urea can be found in a comparison of the concentration of urea in the blood with the rate of excretion in the urine, and that it is possible to express this relationship numerically by the so-called "Ambard's coefficient."

In the calculation of Ambard's coefficient the following factors are taken into account, and they are represented in the formula by the symbols indicated:

D. = *Urea grams excreted in urine in twenty-four hours.* This is obtained as follows: (1) Give the patient 150 to 200 c.c. of water. (2) One-half hour afterward have him empty his bladder and note the time of completion to the *minute.* (3) Discard this urine. (4) At the end of an exactly measured period, preferably two hours, have the patient again empty the bladder completely. (5) Measure this urine exactly and estimate its urea. (6) From this calculate the number of grams of urea which would be excreted in twenty-four hours.

C. = *Urea grams in* 1000 *c.c. of urine.* This is calculated from the above estimation.

Ur. = *Urea grams in* 1000 *c.c. of blood.* The blood is taken from a vein in the middle of the period during which urine is collected, and urea is estimated as described on page 337.

Wt. = *Weight of patient, without clothing, in kilograms.*

The above factors are combined in the following formula, in which the figure 70 is the standard normal body weight in kilograms and 25 is the standard amount of urea in grams per 1000 c.c. of urine.

$$\frac{\text{Ur}}{\sqrt{\text{D} \times \frac{70}{\text{Wt}} \times \sqrt{\frac{\text{C}}{25}}}} = \text{Coefficient.}$$

With normal kidneys a coefficient of 0.06 to 0.09 is obtained, regardless of how high the blood nitrogen may rise from diet or other causes. When the coefficient rises above 0.09 an impairment of the power of the kidneys to excrete urea is to be inferred. The extent to which it rises in different grades of impairment is shown in the table on page 97.

McLean's index is based upon Ambard's coefficient and is perhaps more widely used in this country. It expresses the same relationship in a somewhat more satisfactory manner in that it adopts 100 as the

normal index, and this falls in proportion to the degree of kidney insufficiency instead of rising, as is the case with Ambard's coefficient. McLean's formula is as follows: $\frac{D \times \sqrt{C} \times 8.96}{Wt \times Ur^2} = \text{Index.}$

McLean has constructed a slide rule, by means of which it is possible with the given data to work out the index in a few minutes without calculation.

As a test of renal function Ambard's coefficient, both in its original form and in the form of McLean's index, has received wide attention. It has been shown to parallel the phenolsulphonephthalein test with striking constancy and, like it, to show kidney derangement in cases of cardiac decompensation with congestion of the kidneys in which there is little or no nitrogen retention. In general, it is regarded as a valuable guide. At its best, however, it probably gives no information not offered by other and very much simpler tests.

III. CHEMICAL EXAMINATION

Methods

Qualitative and quantitative methods for the individual substances are given in their appropriate places. It is convenient at this place to include a general description of colorimetric and centrifugal methods which have rather wide usefulness for quantitative estimations. Their application to individual substances will be given later.

Colorimetric Methods.—These combine comparative simplicity and great accuracy and are steadily growing in popularity.

In general, they consist in treating the fluid under examination with such reagents as will produce a soluble colored compound with the substance to be estimated, and in comparing this color with that of a similar solution of known strength, upon the principle that the depth of color is directly proportionate to the amount of the substance present. Some preliminary treatment is usually necessary to remove interfering substances. Any device which will show the quantitative relationship between the colors is called a colorimeter.

The chief hindrances to the wide adoption of colorimetric methods for clinical purposes are the cost of the colorimeter and the difficulties in the way of preparing standard color solutions. Relatively stable standard solutions for many of the methods can now be purchased ready prepared.

The **Duboscq type** of colorimeter, including the original Duboscq, the Kober, and certain others manufactured in this country, represents the highest type of colorimeter available for clinical work.

The general construction is well shown in Figure 36. The solutions to be compared are placed in glass-bottomed cups, which can be raised by means of rack and pinion until the lower ends of the clear glass plungers are immersed in the fluid, the excess of fluid rising between the plungers and the walls of the cups. By raising or lowering the cups the layer of fluid between the lower ends of the plungers and the bottom of the cups may be made of any desired thickness, and the thickness of each is indicated by a scale placed in a convenient position. Beneath

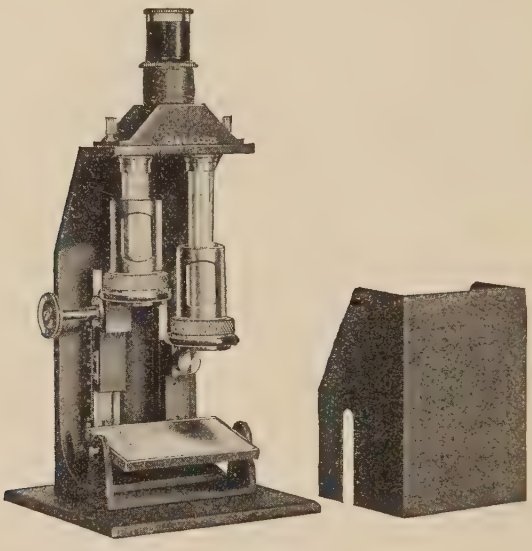


FIG. 36.—The biologic colorimeter, a small colorimeter of the Duboscq type, which is very satisfactory for clinical laboratory work. The light-shield has been removed to show the cups.

the cups is a mirror which reflects light up through the cups and the long axis of the plungers into a series of prisms. These reflect the light from the two cups into a single field which is viewed by an eye-lens. Each lateral half of the field receives its light through one of the cups. The raising or lowering of the cups, by diminishing or increasing the thickness of the layer of fluid through which the light passes, diminishes or increases the depth of color of the corresponding half of the field.

If identical fluids are poured into both cups and the cups placed at the same height, then the two halves of the field should have exactly the same depth of color. If they do not exactly match, the scale, which is movable upon most instruments, must be brought to accurate adjust-

ment. In the absence of an adjustable scale a correction must be made in the height of the cup which is to receive the standard—customarily the left-hand cup. If, for example, with the right cup at 10 mm., the reading of the left is 10.2 mm., then the left, which is to contain the standard solution, should be set at 10.2 when making the tests.

To use the colorimeter, focus the eye-piece and arrange the reflector so that the two halves of the field are equally illuminated. Fill one of the cups half full of the standard color solution and raise this cup until the layer of fluid between the bottom of the cup and the lower end of the plunger is of a convenient thickness. This will usually be 10 or 20 mm., as indicated by the scale. Place the unknown solution in the other cup and move this cup up and down until the two halves of the field viewed by the eye-piece exactly match in color. Either daylight or artificial light which is filtered through daylight glass may be used. With daylight, readings are most accurate when the colorimeter is placed in front of a window, but far enough from it—6 to10 feet—to avoid any strong light entering the eye. Artificial light is best used in a darkened room or a dark corner of the laboratory. Note the reading on the scale. The concentrations of the two solutions are inversely proportional to the respective readings when the colors match. This may be expressed in the formula:

$$\text{Strength of unknown} = \frac{\text{Reading of standard} \times \text{Strength of standard}}{\text{Reading of unknown}}$$

If, for example, in the phenolsulphonephthalein test the 50 per cent. standard be used, while the cup containing it is placed at 10 mm., and the unknown stands at 15 mm. when the colors match, then

$$\text{Strength of unknown} = \frac{10 \times 50}{15} = 33.3 \text{ per cent.}$$

Special formulæ for blood-chemical methods are given in Chapter III.

Results are always most accurate when the unknown and the standard have nearly the same depth of color.

The **Bock-Benedict colorimeter** employs the same general principle as the Duboscq. The standard solution is placed in a glass trough of standard thickness, while the unknown is placed in a cup with plunger.

The **Klett-Bio colorimeter** (Fig. 37) is a new colorimeter with several unique features, such as a built-in substage lamp and a revolving calculation table. The principle of operation is essentially the same as that of the Duboscq colorimeter.

The **Hellige colorimeter** (Fig. 38), devised by Autenrieth and Koenigsberger, but sold under the name of the manufacturer, is less accurate than the Duboscq, but is very satisfactory for certain purposes, particularly the phenolsulphonephthalein test. The solution under examination is placed in the box or trough, B, while the standard solution is placed in the wedge-shaped bottle, C, which can be moved

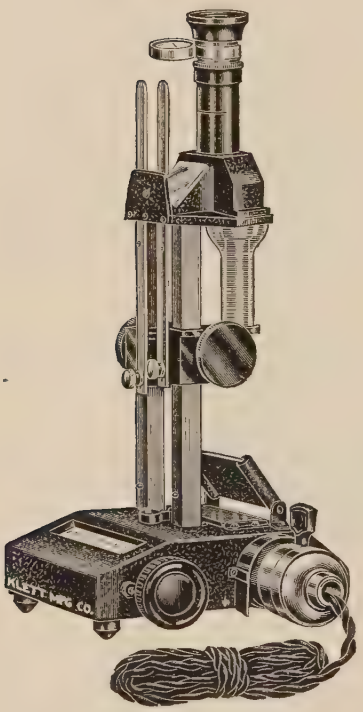


Fig. 37.—The Klett-Bio colorimeter.

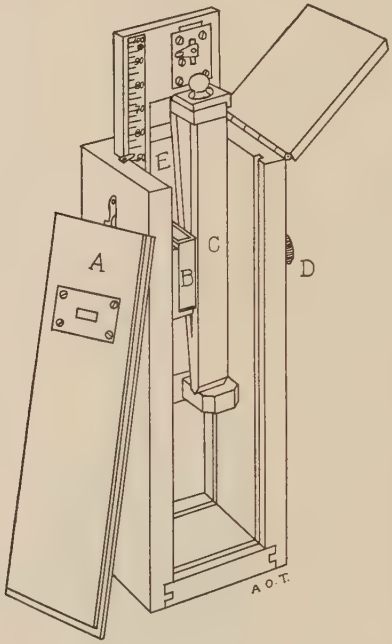


Fig. 38.—Hellige colorimeter: *A*, Sliding front, removed. It has a small window through which the colored solutions in *B* and *C* are viewed. A prism behind the window brings the two colors close together. *B*, Rectangular glass box for unknown solution; *C*, glass wedge-shaped bottle for standard solution; *D*, knerled head by means of which the back-board with the wedge (*C*) and the scale are moved up and down; *E*, ground-glass back.

up or down beside the trough. The front, A, is slipped into place, and the two solutions are viewed through the window behind which is a double prism to bring the two colors close together. The wedge is moved up and down by means of the knerled head, D, until a point is reached where the two colors match. The figure on the scale which then stands opposite the pointer indicates the relation between the strengths of the two solutions. If the pointer stands at 40, then the

unknown solution is 40 per cent. as strong as the known standard; if at 70, it is 70 per cent. as strong. Hermetically sealed standard wedges for most of the tests can be purchased with the instrument.

Before the instrument is used its accuracy should be tested. The following plan is simple: Place the same colored solution—for example, a phenolsulphonephthalein solution—in both the wedge and the glass box, and rack the wedge up and down until the colors match. The scale should then read 100. Now dilute the solution in the glass box exactly 1 in 10 (1 part of solution, 9 parts of water). This should give

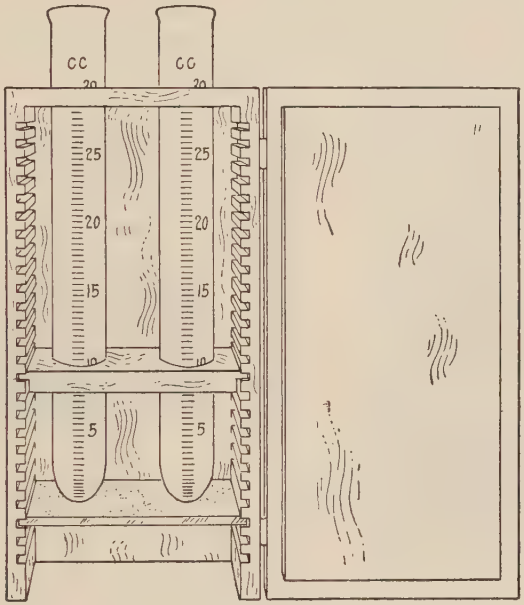


Fig. 39.—Denison laboratory colorimeter, made from a slide box, blackened inside, and two 30-c.c. tubes which stand upon a ground-glass slide and are held in place by a wooden slide.

a reading of 10 when the colors match. Test the intermediate graduations of the scale in the same way. If the scale proves to be inaccurate, make a record of the figures to be added or subtracted.

The **Denison laboratory colorimeter**[1] (Fig. 39) is one of the simplest, most convenient, and least expensive yet devised, and is sufficiently accurate for most clinical purposes, including ordinary blood-chemical determinations. The instrument can be easily made by any one from a Pillsbury slide box and two graduated 30-c.c. test-tubes.

[1] Designed by the late A. R. Peebles, while director of the Denison Research Laboratory, University of Colorado.

These tubes are carried in stock by most supply houses, and answer as well as specially graduated tubes. Equivalent graduations on the two tubes must stand at the same height.

To use the instrument the unknown solution is pipeted into one tube exactly to the 10-c.c. mark, the reading being taken at the bottom of the meniscus, and the standard solution is placed in the other, a little at a time by means of a capillary pipet, until the colors in the two tubes just match when viewed from above over a sheet of white paper or a small mirror, so placed that it reflects the light from a window. A small reflector can be placed in the bottom of the box at an angle of 45 degrees if desired, but adds to the cost without commensurate advantage. When the two colors match, the height of the standard color solution expressed

FIG. 40.—The Purdy electric centrifuge with four arms.

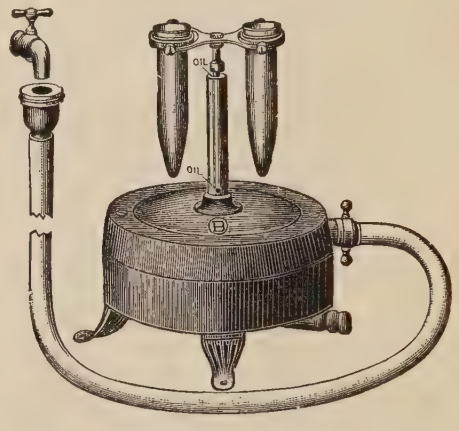


FIG. 41.—Water-motor centrifuge. This instrument is very satisfactory for a small laboratory, as it is inexpensive, requires little care, and if kept oiled lasts almost indefinitely.

in tenths of a cubic centimeter will indicate in percentage the relation between the strengths of the two solutions. If, for example, the top of the standard solution stands at the 7.5 c.c. mark, then the unknown solution is 75 per cent. as strong as the known standard. When desired the instrument may be used in the same manner as the Duboscq, placing the standard solution to a desired height, usually to the 10-c.c. mark, and varying the amount of the unknown until the colors match. In this case all the formulæ given for calculation with the Duboscq may be used. Readings are most accurate when the unknown solution and the standard have nearly the same depth of color and are best made in front of a window and at some distance—5 to 10 feet—from it.

Centrifugal Methods.—As shown by Purdy, the centrifuge offers

a means of making quantitative estimations of a number of substances in the urine. Results are easily and quickly obtained; and while the methods can lay no claim to accuracy, they will be found very useful in following the progress of a case from day to day when recourse to more elaborate methods is out of the question.

In general, the methods consist in precipitating the substance to be estimated in a graduated centrifuge tube by means of an appropriate reagent, and applying a definite amount of centrifugal force for a definite length of time, after which the volume of precipitate is read off upon the side of the tube. Interfering substances must be previously removed. Results are in terms of *bulk of precipitate*, which must not be confused with *percentage by weight*. The weight percentage can be found by referring to Purdy's tables, given later; but in following the progress of the same case from day to day it suffices to compare the bulk of the precipitate, always taking into consideration, of course, the twenty-four-hour amount of urine.

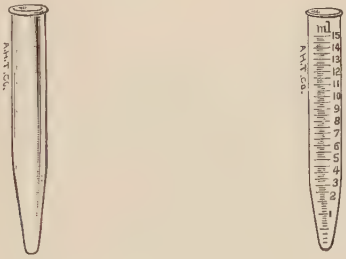


FIG. 42.—Conical 15-c.c. centrifuge tubes, plain and graduated. A narrow tip is desirable.

To fulfil Purdy's requirements, upon which the tables are based, the centrifuge should have an arm with a radius of $6\frac{3}{4}$ inches when in motion, and should be capable of maintaining a speed of 1500 revolutions a minute. The electric centrifuge is to be recommended, although good work can be done with a water-power centrifuge or, after a little practice, with the hand centrifuge. A speed indicator is desirable with electric and water-motor machines, although one can learn to estimate the speed by the musical note. In general, a four-arm centrifuge will be found most useful. Instead of the conical aluminum tube-shields usually supplied, it is well to get flat-bottomed shields with rubber cushions, because these permit the use of ordinary test-tubes, which is a great convenience at times. When the centrifuge is in use opposite tubes must carry the same weight, otherwise the machine will be quickly ruined. It is best to balance the filled tubes upon a scale, but it will usually suffice to fill them to the same height.

A. Normal Constituents

Of the large number of organic and inorganic substances normally present in the urine, only a few demand any consideration from the clinician. The table given below therefore outlines the average composition from the clinical, rather than from the chemical, standpoint. Only the twenty-four-hour quantities are given, since they alone furnish an accurate basis for comparison. *The student cannot too soon learn that percentages mean little or nothing except as they furnish a means of calculating the twenty-four-hour elimination.*

Although the conjugate sulphates are organic compounds, they are, for the sake of convenience, included with the inorganic sulphates in the table.

COMPOSITION OF NORMAL URINE

	Grams in twenty-four hours.	Grams, approximate average.
Water	1000–1500	1200
Total substances in solution	55–70	60
Inorganic substances	20–30	25
Chlorids (chiefly sodium chlorid)	10–15	12.5
Phosphates (estimated as phosphoric acid), total	2.5–3.5	3
Earthy, one-third of total		1
Alkaline, two-thirds of total		2
Sulphates (estimated as sulphuric acid), total	1.5–3	2.5
Mineral, nine-tenths of total		2.25
Conjugate, one-tenth of total		0.25
Includes indican		Trace
Ammonia	0.5–1	0.7
Organic substances	30–40	35
Urea	25–35	30
Uric acid	0.4–1	0.7

Among constituents which are of little clinical importance, or are present only in traces, are:

Inorganic.—Iron, carbonates, nitrates, silicates, and fluorids.

Organic.—Creatinin, hippuric acid, purin bases, oxalic acid, volatile fatty acids, pigments, and acetone.

Variations in body weight, diet, and exercise cause marked fluctuations in the total solids and in individual substances.

1. Chlorids.—These are derived from the food, and are mainly in the form of sodium chlorid. The amount excreted normally is 10 to 15 gm. in twenty-four hours. It is much affected by the diet, and is reduced to a minimum in starvation.

Excretion of chlorids is diminished in some forms of nephritis and in fevers, especially in pneumonia and inflammations, leading to the formation of large exudates. In nephritis, particularly the form designated chronic parenchymatous nephritis, the kidneys are less permeable to the chlorids; and it is possible that the edema is due largely to an effort of the body to dilute the chlorids which have been retained. Certainly an excess of chlorids in the food will in many cases increase both the albuminuria and the edema of nephritis. In fevers the diminution is due largely to decrease of food, though probably in some measure to impaired renal function. In pneumonia chlorids are constantly very low, and in some cases are absent entirely. Following the crisis they are increased. In inflammations leading to formation of large exudates—for example, pleurisy with effusion—chlorids are diminished because a considerable amount becomes "locked up" in the exudate. During absorption chlorids are liberated and appear in the urine in excessive amounts.

Diminution of chlorids is also sometimes observed in severe diarrhea, anemic conditions, and carcinoma of the stomach.

Detection of Chlorids.—The following simple test will show the presence of chlorids, and at the same time roughly indicate any pronounced alteration in amount. It is especially useful in following the excretion of chlorids in cases of pneumonia:

To a few cubic centimeters of albumin-free urine in a test-tube add a few drops of nitric acid to prevent precipitation of phosphates and then a few drops of silver nitrate solution of about 12 per cent. strength. A white, curdy precipitate of silver chlorid forms. If the urine merely becomes milky or opalescent, chlorids are markedly diminished.

Quantitative Estimation.—The well-known and reliable Volhard method has been simplified by Strauss, and this modification has been still further simplified by Bayne-Jones and by McLean and Selling, so that the method is now available for ordinary clinical work. The only difficulty is the preparation of solutions, and these can be purchased ready prepared. A much less accurate though simple and very useful method is afforded by the centrifuge.

1. **Simplified Volhard Method.**—As a rule, albumin need not be removed. In an accurately graduated 50-c.c. cylinder place 5 c.c. of

the urine and 10 c.c. of Solution No. 1. Mix by inverting several times. If a reddish color appears, add 3 drops of 10 per cent. potassium permanganate. After five minutes add Solution No. 2, a very little at a time, mixing after each addition, until a permanent reddish-brown color (best seen against a white background) appears. This is the end-point.

The solutions are so balanced that if the urine be chlorid free the volume of fluid when the end-point is reached will be 35 c.c., and that for each gram per liter of chlorids in the urine the volume will be 1 c.c. less. Therefore, the difference between 35 c.c. and the height of the fluid at the end of the test gives directly the number of grams of chlorids per liter of urine, expressed as sodium chlorid. If, for example, the fluid reaches the 28-c.c. mark, 35 − 28 = 7 gm. of sodium chlorid per liter of urine.

A certified 50-c.c. graduated cylinder, with glass stopper, is required. The ordinary 50-c.c. graduate is not sufficiently accurate.

The **solutions** are as follows:

No. 1.—*Standard silver nitrate solution:*

Silver nitrate (C. P., anhydrous, crystallized)	29.055 gm.
Nitric acid (25 per cent.)	900 c.c.
Ammonioferric alum (cold saturated solution)	50 "
Distilled water to	1000 "

No. 2.—*Ammonium sulphocyanate solution:*

Ammonium sulphocyanate	7 gm.
Distilled water	1000 c.c.

This solution is intentionally made too strong, and it must be standardized by diluting with distilled water until exactly 20 c.c. (and no less) will produce a red color when mixed with exactly 10 c.c. of Solution No. 1.

2. **Centrifugal Method.**—Fill a graduated centrifuge tube to the 10-c.c. mark with urine; add 15 drops strong nitric acid and then silver nitrate solution of 12 per cent. strength to the 15-c.c. mark. Mix by inverting several times. Let stand a few minutes for a precipitate to form, and then revolve in the centrifuge at about 1200 revolutions a minute for three minutes or until the bulk of the precipitate remains constant. Read off the volume of precipitated silver chlorid and convert this into percentage by weight of chlorin or sodium chlorid by means of the table on page 117.

2. Phosphates are derived largely from the food, only a small proportion resulting from metabolism. The normal daily output of phosphoric acid is about 2.5 to 3.5 gm.

The urinary phosphates are of two kinds: *alkaline*, which make

PURDY'S CENTRIFUGAL METHOD FOR CHLORIDS

Table showing the relation between the volume of precipitate of silver chlorid (AgCl) after centrifugation and the gravimetric percentage of sodium chlorid (NaCl) and chlorin (Cl).

Volume of precipitate of AgCl in c.c.	Percentage NaCl.	Percentage Cl.	Volume of precipitate of AgCl in c.c.	Percentage NaCl.	Percentage Cl.
0.1	0.13	0.08	0.9	1.17	0.71
0.2	0.26	0.16	1.0	1.3	0.79
0.3	0.39	0.24	1.2	1.50	0.95
0.4	0.52	0.32	1.4	1.82	1.1
0.5	0.65	0.39	1.6	2.07	1.26
0.6	0.78	0.47	1.8	2.33	1.42
0.7	0.91	0.55	2.0	2.59	1.58
0.8	1.04	0.63			

up two-thirds of the whole, and include the phosphates of sodium and potassium, and *earthy*, which constitute one-third, and include the phosphates of calcium and magnesium. Earthy phosphates are frequently thrown out of solution in neutral and alkaline urines, and as "amorphous phosphates" form a very common sediment. This sediment seldom indicates an excessive excretion of phosphoric acid. It is usually merely an evidence of diminished acidity of the urine, or of an increase in the proportion of phosphoric acid eliminated as earthy phosphates. This form of "phosphaturia" is most frequent in neurasthenia and hysteria. When the urine undergoes ammoniacal decomposition some of the ammonia set free combines with magnesium phosphate to form ammoniomagnesium phosphate ("triple phosphate"), which is only slightly soluble in alkaline urine and is deposited in typical crystalline form (p. 180).

From the clinical point of view variations in the amount of phosphates in the urine are unimportant and no method for their determination need be given here.

3. Sulphates.—The urinary sulphates are derived partly from the food, especially meats, and partly from body metabolism. The normal output of sulphuric acid is about 1.5 to 3 gm. daily. It is increased in conditions associated with active metabolism, and in general may be taken as a rough index of protein metabolism.

Quantitative estimation of the total sulphates yields little of clinical value and may be omitted.

About nine-tenths of the sulphuric acid is in combination with various mineral substances, chiefly sodium, potassium, calcium, and magnesium (*mineral* or *preformed sulphates*). One-tenth is in combination with certain aromatic substances, which are mostly products of protein putrefaction in the intestine, but are derived in part from destructive metabolism (*conjugate* or *etheral sulphates*). Among these aromatic substances are indol, phenol, and skatol. By far the most important of the conjugate sulphates and representative of the group is potassium indoxyl sulphate.

Potassium indoxyl sulphate, or **indican,** is derived from indol. Indol is absorbed and oxidized into indoxyl, which combines with sulphuric acid and potassium and is thus excreted. Under normal conditions the amount in the urine is small. It is increased by a diet rich in protein.

Unlike the other ethereal sulphates, which are derived in part from metabolism, indican originates practically wholly from putrefactive processes. It alone, therefore, and not the total ethereal sulphates, can be taken as an index of such putrefaction. A marked increase is called *indicanuria* and is often associated with lassitude and headache. Not infrequently it is merely evidence of too much meat in the diet. Pathologically indicanuria is noted in:

(*a*) *Diseases of the Small Intestine.*—This is by far the most common source. Intestinal obstruction gives the largest amounts of indican. It is also much increased in intestinal indigestion—so-called "biliousness"; in inflammations, especially in cholera and typhoid fever; and in paralysis of peristalsis, such as occurs in peritonitis. Simple constipation and diseases of the *large* intestine alone rarely cause indicanuria.

(*b*) *Diseases of the stomach* associated with deficient hydrochloric acid, as chronic gastritis and gastric cancer. Diminished hydrochloric acid favors intestinal putrefaction. Simon has called attention to the rather frequent occurrence of indicanuria in cases of gastric ulcer in which hyperchlorhydria is the rule, a fact which is as yet unexplained.

(*c*) *Diminished Flow of Bile.*—Since the bile serves as a stimulant to peristalsis and in several ways retards putrefaction, a diminished flow from any cause favors occurrence of indicanuria.

(*d*) *Decomposition of exudates* anywhere in the body, as in empyema, bronchiectasis, and large tuberculous cavities.

Detection of indican depends upon its decomposition and subsequent oxidation of the indoxyl set free into indigo-blue. This change sometimes takes place spontaneously in decomposing urine, causing a dirty blue color. Crystals of indigo (Fig. 54) may then be found both in the sediment and the scum.

Obermayer's Method.—Take a test-tube, about one-third full of the urine and add an equal volume of Obermayer's reagent and a few cubic centimeters of chloroform. For best results the proportion of urine and reagent must be closely adhered to and the mixture should be warmed before adding the chloroform. Mix by inverting a few times. Avoid shaking violently as this may emulsify the chloroform. If indican be present in excess, the chloroform, which sinks to the bottom, will assume an indigo-blue color. The indican in normal urine may give a faint blue by this method. The depth of color indicates the comparative amount of indican if the same proportions of urine and reagents are always used, but one should bear in mind the total amount of urine voided. It is well to keep on hand one or two test-tubes with marks indicating the amounts of urine and reagents to be used. An accurate quantitative method is unnecessary in clinical work.

Urine of patients taking iodids gives a reddish-violet color with Obermayer's reagent, and this may obscure even a fairly strong indican reaction. However, upon addition of a few drops of strong sodium hyposulphite solution and shaking, the violet color will disappear, leaving the blue if indican be present. Occasionally, owing to slow oxidation, indigo-red will form instead of indigo-blue. This gives a color much like that due to iodids, but it does not disappear when treated with sodium hyposulphite. A color somewhat similar to that of indigo-blue may be produced by guaiacol, but this soon changes to yellowish green. Hexamethylenamin prevents the reaction even when a large amount of indican is present, as does formaldehyd when added to the urine as a preservative. Bile-pigments, which interfere with the test, must be removed if present (p. 85).

Obermayer's reagent consists of strong hydrochloric acid (sp. gr. 1.19), 1000 c.c., and ferric chlorid, 2 gm. This makes a yellow, fuming liquid which keeps well.

4. Urea.—From the standpoint of physiology urea is the most important constituent of the urine. It is the principal waste-product of metabolism, and constitutes about one-half of all the solids excreted—about 20 to 35 gm. in twenty-four hours. It

represents 85 to 90 per cent. of the total nitrogen of the urine, and its quantitative estimation is a simple, though not very accurate, method of ascertaining the state of nitrogenous excretion. This is true, however, only in normal individuals upon average mixed diet. On a low protein diet it may fall to 60 per cent. of the total nitrogen. Under pathologic conditions the proportion of nitrogen distributed among the various nitrogen-containing substances undergoes great variation. The only accurate index of protein metabolism is, therefore, the total output of nitrogen, which can be estimated by the Kjeldahl method or one of its modifications. The whole subject of "nitrogen partition" (distribution of nitrogen among the nitrogen-containing bodies) and "nitrogen equilibrium" (relation of excretion to intake) is an important one, but is out of the province of this book, since as yet it concerns the biochemist more than it does the clinician.

It may be helpful to state here, however, that upon a mixed diet the nitrogen of the urine is distributed about as follows: Urea nitrogen, 86.9 per cent.; ammonia nitrogen, 4.4 per cent.; creatinin nitrogen, 3.6 per cent.; uric acid nitrogen, 0.75 per cent.; "undetermined nitrogen," chiefly in amino-acids, 4.3 per cent.

Normally, the amount is greatly influenced by exercise and diet. It is increased by copious drinking of water and administration of ammonium salts of organic acids.

Pathologically, urea is increased in fevers, in diabetes when acidosis is not marked, and especially during resolution of pneumonia and absorption of large exudates. As above indicated, when other factors are equal, the amount of urea indicates the activity of metabolism. In deciding whether in a given case an increase of urea is due to increased metabolism the relation between the amounts of urea and of the chlorids is a helpful consideration. On a mixed diet the amount of urea is normally about twice that of the chlorids. If the proportion is much increased above this, increased tissue destruction may be inferred, since other conditions which increase urea also increase chlorids.

In general, a pathologic decrease in amount of urea is due either to lessened formation within the body or to diminished excretion. *Decreased formation* of urea occurs in diseases of the liver with des-

truction of liver substance, such as marked cirrhosis, carcinoma, and acute yellow atrophy. The state of acidosis likewise decreases formation of urea, because nitrogen which would otherwise be built into urea is eliminated in the form of ammonia (p. 127). *Retention* of urea occurs in most cases of nephritis. In acute nephritis the amount of urea in the urine is markedly decreased, and a return to normal denotes improvement. In the early stages of chronic nephritis, when diagnosis is difficult, it is usually normal. In the late stages, when diagnosis is comparatively easy, it is decreased. Hence estimation of urea is of little help in the diagnosis of this disease, and is of no value whatever when, as is so frequently the case, a small quantity of urine taken at random is used. When, however, the diagnosis is established, estimations made at frequent intervals under the same conditions of diet and exercise are of much value, *provided a sample of the mixed twenty-four-hour urine be used*. A steady decline is a very bad prognostic sign, and a sudden marked diminution is usually a forerunner of uremia. Much more helpful in the study of nephritis is the estimation of the extent of retention of urea in the blood, which is discussed on pages 104 and 211. Estimations of urea in urine are, in fact, made much less frequently than formerly.

The presence of urea can be shown by allowing a few drops of the fluid partially to evaporate upon a slide, and adding a small drop of pure, colorless nitric acid or saturated solution of oxalic acid. Crystals of urea nitrate or oxalate (Fig. 43) will soon appear and can be recognized with the microscope.

Quantitative Estimation.—The hypobromite method, which has long been used in clinical work, is very simple, but is notoriously inaccurate, since it gives more nearly the total nitrogen than the urea. It is, however, of much value in comparing urine secured from the two kidneys separately by ureteral catheterization. The new urease methods are much more accurate.

1. Hypobromite Method.—This depends upon the fact that urea is decomposed by sodium hypobromite with liberation of nitrogen. The amount of urea is calculated from the volume of nitrogen set free. Of the many forms of apparatus devised for this purpose, that of Doremus-Hinds (Fig. 44) is probably the most convenient.

Pour some of the urine into the smaller tube of the apparatus, then open the stop-cock and quickly close it so as to fill its lumen with urine.

Rinse out the larger tube with water and fill it and one-half of the bulb with 25 per cent. caustic soda solution. Add to this 1 c.c. of bromin by means of a medicine-dropper and mix well. This prepares a fresh solution of sodium hypobromite with excess of caustic soda, which serves to absorb the carbon dioxid set free in the decomposition of urea. When handling bromin keep an open vessel of ammonia near to neutralize the irritant fumes.

Pour the urine into the smaller tube, and then turn the stop-cock so as to let as much urine as desired (usually 1 c.c.) run slowly into the hypobromite solution. When bubbles have ceased to rise, read off the height of the fluid in the large tube by the graduations upon its side. This gives the amount by weight of urea in the urine added, from which the amount excreted in twenty-four hours can easily be calculated. If

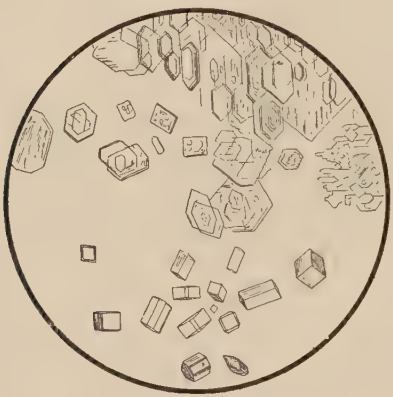


FIG. 43.—Crystals of nitrate of urea (upper half) and oxalate of urea (lower half) (after Funke).

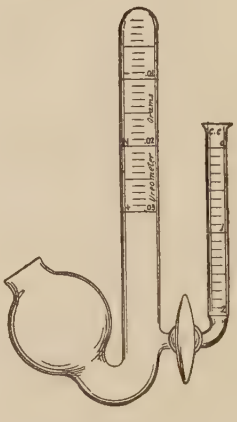


FIG. 44.—Doremus-Hinds' ureometer without foot.

the urine contains much more than the normal amount, it should be diluted. Albumin, if present, must be removed by acidifying with acetic acid, boiling, and filtering.

To avoid handling pure bromin, which is disagreeable, Rice's solutions may be employed:

(*a*)	Bromin	31 gm.
	Potassium bromid	31 "
	Distilled water	250 c.c.
(*b*)	Sodium hydroxid	100 gm.
	Distilled water	250 c.c.

Equal parts of these solutions are mixed and used for the test. The bromin solution must be kept in a tightly stoppered bottle or it will rapidly lose strength.

The hypobromite method has fallen into disrepute largely because of inconstant results, and because it gives more nearly the total nitrogen than the urea owing to decomposition of other nitrogenous constituents. According to Robinson and Müller, the discrepancies are due to insufficient mixing of urine and hypobromite and can be obviated by gentle shaking after the first vigorous reaction is over. Results are then constant, but too high for urea, and for clinical purposes may be taken as representing the total nitrogen of the urine, in which case the urea figure is divided by 2.14 to reduce it to terms of nitrogen.

2. **Urease Methods.**—These are based upon the conversion of urea into ammonium carbonate by urease, a ferment first extracted by Takeuchi from the soy bean in 1909. The urea is estimated from the amount of ammonium carbonate produced by the fermentation. There are several clinical methods, two of which are here given in detail. In the first, the urine after fermentation is titrated with decinormal hydrochloric acid in the presence of the indicator, methyl orange. It is not entirely accurate, but is much superior to the hypobromite method. In the second the ammonia is determined by nesslerization. This method is sufficiently accurate for the most exacting work, but is too complicated for use in a physician's office laboratory. It is included here because of the growing importance of exact estimations of the various nitrogenous substances in blood and urine, and because of the growing popularity of colorimetric methods.

Neither albumin nor sugar nor any other substance likely to be present in body fluids interferes with the action of urease.

Marshall's Urease Method.—1. Into each of two 200-c.c. flasks measure 5 c.c. of the urine and about 100 c.c. of water, and to one add 1 c.c. of 5 per cent. solution of urease.[1]

2. Overlay the fluid in each flask with about 1 c.c. of toluol, insert corks, and let stand over night at room temperature (or for three hours in the incubator at 37° C.).

3. At the end of this time titrate the contents of each flask to a distinct pink color with decinormal hydrochloric acid, using a few drops of 0.5 per cent. methyl orange solution as indicator.

4. Find the difference between the number of cubic centimeters of decinormal acid used in the two titrations and multiply this by the factor 0.06 to obtain the percentage of urea in the urine. From the percentage calculate the twenty-four-hour elimination.

[1] Urease powder with the necessary phosphate added to increase its activity is prepared by the Arlington Chemical Co. and E. R. Squibb & Sons. The physician will find it more convenient to use the 0.025-gm. tablets sold by Hynson, Westcott & Dunning, Baltimore. Two of these are crushed and dissolved in 5 c.c. of water, and the whole of this solution is used for the test.

Urease Method of Folin and Youngburg.—*Reagents Required.*—(*a*) Permutite powder (see footnote, p. 129).

(*b*) Urease solution, pyrophosphate solution, Nessler's reagent, and standard ammonium sulphate solution as described for blood urea on page 337.

(*c*) Ammonia-free distilled water must be used throughout. Water may be freed of its ammonia by shaking with permutite powder and decanting.

Method.—1. Remove ammonia from the urine as follows: Carefully dilute 5 c.c. of the mixed twenty-four-hour urine to 50 c.c., or 10 c.c. to 50 c.c. if the specific gravity be very low. Mix well and filter. Place about 25 c.c. of the diluted urine in a 200-c.c. flask with about 4 gm. of dry permutite powder and agitate gently for five minutes. Allow to settle for fifteen to thirty seconds or centrifugalize and decant the clear fluid.

2. Place exactly 1 c.c. of the diluted ammonia-free urine in a test-tube and add 1 c.c. of urease solution and 1 drop of the pyrophosphate solution.

3. Place in a large beaker of water at 40° to 55° C. for five minutes, or let stand at room temperature for fifteen minutes.

4. Transfer the fluid to a 200-c.c. volumetric flask, rinsing the tube with distilled water and adding the rinsings to the flask, and dilute to about 150 c.c.

5. At this stage prepare the standard by placing in another 200-c.c. volumetric flask 10 c.c. of the standard ammonium sulphate solution (representing 1 mg. of nitrogen), 1 c.c. of urease solution, and about 140 c.c. of water.

6. Add 20 c.c. of Nessler's reagent to each flask as nearly simultaneously as possible, dilute to the 200 c.c. marks with distilled water, and mix well. In adding the Nessler's reagent give the flask a whirl and run in the reagent from a pipet while the fluid is still whirling.

7. Compare the unknown with the standard in a colorimeter. The calculation is based upon the fact that the unknown represents 0.1 or 0.2 c.c. of urine, depending upon the dilution employed, while the standard contains 1 mg. of nitrogen. With the Duboscq and Denison Laboratory colorimeters the following formula may be used, D representing the dilution of the urine, that is, the number of cubic centimeters to which 1 c.c. of the urine was diluted in Step 1:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{D} \times 100 = \text{mg. urea-nitrogen in 100 c.c. urine.}$$

8. It is generally desirable to record results in terms of urea instead of urea-nitrogen. To this end multiply the figure for urea-nitrogen by 2.14.

5. Uric acid is the most important of a group of substances, called *purin bodies*, which are derived chiefly from the nucleins of the food, *exogenous uric acid*, and from metabolic destruction of the nuclei of the body, *endogenous uric acid*. The daily output of uric acid is about 0.4 to 1 gm. The amount of the other purin bodies together is about one-tenth that of uric acid. Excretion of these substances is greatly increased by a diet rich in nucleins, as sweetbreads and liver.

Uric acid exists in the urine in the form of urates, chiefly of sodium and potassium, which in concentrated urines are readily thrown out of solution and constitute the familiar sediment of "amorphous urates." This, together with the fact that uric acid is frequently deposited as crystals, constitutes its chief interest to the practitioner. It is a very common error to consider these deposits as evidence of excessive excretion.

Pathologically, the greatest increase of uric acid occurs in leukemia, where there is extensive destruction of leukocytes, in diseases with active destruction of the liver and other organs rich in nuclei, and during absorption of a pneumonic exudate. There is generally an increase during *x*-ray treatment. Uric acid is decreased before an attack of gout and increased for several days after it, but its etiologic relation is still uncertain. An increase is also noted in acute fevers.

Quantitative Estimation of Purin Bodies.—There is no accurate method which is simple enough for clinical purposes. Of clinical methods, the one given here is perhaps most satisfactory. The urine must be albumin-free.

Cook's Method.—In a centrifuge tube take 10 c.c. urine and add about 1 gm. (about 1 c.c.) sodium carbonate and 1 or 2 c.c. strong ammonia. Shake until the soda is dissolved. The earthy phosphates will be precipitated. Centrifugalize thoroughly and pour off all the clear fluid into a graduated centrifuge tube. To this fluid add 2 c.c. ammonia and 2 c.c. ammoniated silver nitrate solution. The purin bodies will be precipitated as silver compounds. Let stand a few minutes, and revolve in the centrifuge until the bulk of precipitate *remains constant.* Each 0.1 c.c. of sediment represents 0.001176 gm. purin bodies.

Ammoniated silver nitrate solution is prepared by dissolving 5 gm. of silver nitrate in 100 c.c. distilled water, and adding ammonia until the solution clouds and again becomes clear.

Quantitative Estimation of Uric Acid.—Ruhemann's method, while far from accurate, will probably answer for many clinical purposes. The Benedict and Franke method is too complicated for ordinary clinical work, but is given because of the growing importance of accurate colorimetric methods. The estimation is, however, seldom of any direct clinical value.

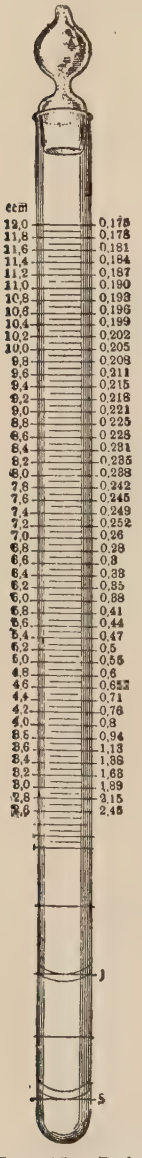


FIG. 45.—Ruhemann's uricometer.

Ruhemann's Method for Uric Acid.—The urine must be slightly acid. By means of a pipet fill Ruhemann's tube (Fig. 45) to the mark S with the indicator, carbon disulphid, so that the lowest part of the meniscus is on a level with the mark, as indicated in Figure 45. Next add Ruhemann's reagent until the base of the upper arch of the meniscus is level with the mark J. The carbon disulphid will assume a violet color. Add the urine, a small quantity at a time, closing the tube with the glass stopper and shaking vigorously after each addition, until the disulphid loses every trace of its violet color and becomes pure white. This completes the test. Toward the end the reagent should be added a very little at a time, and the shaking should be prolonged in order not to pass the end-point. The figure in the right-hand column of figures corresponding to the top of the fluid gives the amount of uric acid in parts per thousand. The presence of diacetic acid interferes with the test, as do also, to some extent, bile and albumin. Diacetic acid can be driven off by boiling; bile-pigment and albumin are removed as described elsewhere (pp. 85 and 147).

Ruhemann's reagent consists of iodin, 0.5 gm.; potassium iodid, 1.25 gm.; absolute alcohol, 7.5 gm.; glycerol, 5 gm.; distilled water to 100 c.c.

Method of Benedict and Franke.—*Reagents Required.*—(*a*) Standard uric acid solution. This is prepared from Benedict and Hitchcock's solution (p. 341) as follows:

Place 50 c.c. of the stock solution, containing 10 mg. uric acid, in a 500-c.c. volumetric flask, add about 350 c.c. water and 25 c.c. of diluted hydrochloric acid (concentrated hydrochloric acid 1 part, water 9 parts). Make up to 500 c.c. with water and

mix well. This solution contains 0.2 mg. uric acid in 10 c.c., and remains good for at least two weeks.

(*b*) Sodium cyanid solution, 5 per cent. This should be freshly prepared about once in two months.

(*c*) Benedict's uric acid reagent, described on page 342.

Method.—Albumin if present must be removed by adding a drop of acetic acid, boiling and filtering. Other substances do not interfere.

1. Dilute the filtered urine with water so that 10 c.c. will contain between 0.15 and 0.30 mg. of uric acid. The usual dilution is 1 in 20.

2. Place 10 c.c. of the diluted urine in a 50-c.c. volumetric flask and add 5 c.c. of the 5 per cent. sodium cyanid from a buret. Mark this flask *U*.

3. In another 50-c.c. volumetric flask place 10 c.c. of the standard uric acid solution, containing 0.2 mg. uric acid, and add 5 c.c. of the 5 per cent. sodium cyanid solution.

4. To each flask add 1 c.c. of the uric acid reagent and mix.

5. Let stand five minutes, dilute to the 50 c.c. mark, and mix.

6. Compare the unknown with the standard in a colorimeter. With the Duboscq or Denison Laboratory colorimeter the following formula may be used, D representing the dilution of the urine, that is, the number of cubic centimeters to which 1 c.c. of the urine was diluted:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 2 \times \text{D} = \text{mg. uric acid in 100 c.c. of urine.}$$

6. Ammonia.—A small amount of ammonia, combined with hydrochloric, phosphoric, and sulphuric acids, is always present. Estimated as NH_3, the normal average is about 0.7 gm. in twenty-four hours. This represents 4 to 5 per cent. of the total nitrogen of the urine, ammonia standing next to urea in this respect.

Under ordinary conditions most of the ammonia which results from the metabolic processes is transformed into urea. When, however, acids are present in excess, either from ingestion of mineral acids or from abnormal production of acids within the body (for example, diacetic and oxybutyric acids in diabetes mellitus), ammonia combines with them and is so excreted, the urea of the urine being correspondingly decreased. This is an important part of the mechanism by which the body protects itself against acid intoxication (Acidosis, p. 563). The ammonium salts are not, however, increased in all forms of acidosis, notably not in the acidosis of nephritis.

In diabetes mellitus, and other conditions associated with excessive production of diacetic and oxybutyric acids, the output of ammonium salts is a very important *index of the degree of acidosis.* Ammonia elimination in diabetes with mild acidosis may be 1 or 1.5 gm. daily, rising to 4 or 5 gm. in severe cases and even to 8 or 10 gm. in extreme cases. Ammonia is likewise increased in pernicious vomiting of pregnancy, but *not in nervous vomiting;* and in conditions in which the power to synthesize urea is interfered with, notably cirrhosis and other destructive diseases of the liver and conditions associated with deficient oxygenation. Certain drugs have a marked influence upon ammonia elimination; thus, fixed alkalies and salts of organic acids diminish it, while inorganic acids, such as hydrochloric, increase it.

The ammonia referred to in the above paragraphs is in the form of ammonium salts and should not be confused, as it often is, with the accumulation of free ammonia, which is derived from decomposition of urea after the urine is secreted and which leads to volatile alkalinity.

Quantitative Estimation.—*The urine must be fresh, since decomposition increases the ammonia.* The formalin method is entirely satisfactory for clinical work, though subject to some inaccuracies. When carried out without use of lead acetate it includes amino-acids with the ammonia, hence sometimes gives figures that are too high. The Folin and Bell method gives ammonia only and is accurate. The difference between the figures obtained by the two methods therefore represents amino-acids.

Ronchese-Malfatti Formalin Method.—This depends upon the fact that when formalin is added to the urine the ammonia combines with it, forming hexamethylenamin. The acids with which the ammonia was combined are set free, and their quantity, ascertained by titration with sodium hydroxid, indicates the amount of ammonia.

Take 10 c.c. of the urine in a beaker or evaporating dish, add 50 c.c. water and 10 drops of 0.5 per cent. alcoholic solution of phenolphthalein. Neutralize by adding a weak sodium hydroxid or sodium carbonate solution until a permanent pink color appears. To 5 c.c. formalin add 15 c.c. water and neutralize in the same way. Pour the formalin into the urine. The pink color at once disappears owing to liberation of acids. Now add decinormal sodium hydroxid solution from a buret until the pink color just returns. Each cubic centimeter of the decinormal

solution used in this titration corresponds to 0.0017 gm. of NH_3. This must be multiplied by 10 to obtain the percentage from which the twenty-four-hour elimination of ammonia is calculated.

The method is more complicated, but distinctly more accurate, when carried out as suggested by E. W. Brown: Treat 60 c.c. of urine with 3 gm. of basic lead acetate, stir well, let stand a few minutes, and filter. This removes certain interfering nitrogenous substances. Treat the filtrate with 2 gm. neutral potassium oxalate, stir well, and filter. Take 10 c.c. of the filtrate, add 50 c.c. water and 15 gm. neutral potassium oxalate, and proceed with the ammonia estimation as above outlined.

Permutite Method of Folin and Bell.—*Reagents Required.*—(*a*) Permutite powder.[1]

(*b*) Sodium hydroxid, 10 per cent. aqueous solution.

(*c*) Nessler's reagent and standard ammonium sulphate solution as described for blood urea on page 336.

(*d*) Ammonia-free distilled water must be used throughout.

Method.—1. Place about 2 gm. of permutite powder in a 200-c.c. volumetric flask, add 5 c.c. of water, and mix.

2. Add an exactly measured quantity of urine from the mixed twenty-four-hour specimen, usually 1 or 2 c.c., rinse down the urine with 1 to 5 c.c. of water, and shake gently for five minutes.

3. Rinse the powder from the sides of the flask to the bottom with 25 to 40 c.c. of water and decant, leaving all the powder.

4. Wash the powder by adding about 50 c.c. water, shaking gently, and decanting. In cases of urine rich in bile the washing should be repeated.

5. Add a little water to the powder and then add 5 c.c. of 10 per cent. sodium hydroxid. Mix well, add about 140 c.c. of water, and shake for a few seconds.

6. At this point prepare the standard as follows: In a 200-c.c. volumetric flask place 10 c.c. of the ammonium sulphate solution, representing 1 mg. nitrogen, add 5 c.c. of 10 per cent. sodium hydroxid solution, and about 140 c.c. of water.

7. To each flask add 10 c.c. of Nessler's reagent in the manner described for urea on page 124. Dilute to the 200 c.c. marks with water and mix well.

[1] Permutite is a new synthetic mineral which has strong absorptive power for ammonia. It is manufactured by the Permutite Co., 440 Fourth Avenue, New York. It may be obtained in purified form for laboratory use and of any degree of fineness. Folin and Bell recommend a powder which will pass through a 60-mesh sieve and not through an 80-mesh sieve. When placed in water the powder should settle quickly like sand. Should a fine "dust" remain suspended, it should be removed by washing the powder in several changes of ammonia-free water and drying at room temperature.

8. Compare the unknown with the standard in a colorimeter. Details of the calculation depend upon the type of colorimeter used. With the Duboscq and Denison Laboratory colorimeters the following formula may be used, V representing the number of cubic centimeters of urine employed:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \frac{100}{V} = \text{mg. ammonia-nitrogen in 100 c.c. urine.}$$

9. If, as is generally the case, it is desired to record results in terms of ammonia (NH_3), multiply the figure obtained for ammonia-nitrogen by 1.214.

7. Amylase.—A small quantity of starch-digesting ferment—derived chiefly from the pancreas—can be detected in the urine of healthy persons. According to Brown, under normal conditions the twenty-four-hour urine will digest 1500 to 12,000 c.c. of 1 per cent. starch solution in one-half hour at 38° C. The normal amount of amylase is therefore said to be 1500 to 12,000 units. It is somewhat influenced by the diet.

Amylase of the urine is diminished in pancreatic disease and in nephritis with deficient renal permeability. It is increased in simple obstruction of the pancreatic duct, although as the pancreas becomes involved in the pathologic process the amount diminishes. The estimation of urinary amylase is therefore important in suspected disease of the pancreas, particularly when considered in connection with the pancreatic ferments of the feces. It has also been proposed as a test of renal function, but does not promise much in this field.

Estimation of Amylase.—1. Obtain the twenty-four-hour urine, which must be kept in a cool place and may be preserved by addition of an ounce of toluol. It should be examined without delay.

2. Dilute the urine to 3000 c.c. and mix well.

3. Proceed exactly as described for fecal amylase on page 428, steps 1 to 5, except that a 0.1 per cent. starch solution must be substituted for the 1 per cent. solution recommended for the feces, and a weaker iodin solution must be used. One part of Gram's iodin solution diluted with 4 parts of water will answer.

The normal falls between Tube 8 (1500 units) and Tube 11 (12,000 units).

B. Abnormal Constituents

Those substances which appear in the urine only in pathologic conditions are of much more interest to the clinician than are those which have just been discussed. Among them are: proteins, sugars, the acetone bodies, bile, urobilin, hemoglobin, hematoporphyrin, and the diazo substances. Most of these are present in negligible traces normally. The detection of drugs in the urine will also be discussed under this head.

1. Proteins.—Of the proteins which may appear in the urine, serum-albumin and serum-globulin are the most important. Mucin, proteose, and a few others are found occasionally, but are of less interest.

(1) **Serum-albumin and Serum-globulin.**—These two proteins constitute the so-called "urinary albumin." They usually occur together, have practically the same significance, and both respond to all the ordinary tests for "albumin."

Their presence, or *albuminuria*, is probably the most important pathologic condition of the urine, and also the most frequent. It may be either *accidental* or *renal*. The physician can make no greater mistake than to regard all cases of albuminuria as indicating kidney disease.

Accidental or *false albuminuria* is due to admixture with the urine of albuminous fluids, such as pus, blood, and vaginal discharge. The microscope will usually reveal its nature. It occurs most frequently in pyelitis, cystitis, and chronic vaginitis, and the quantity of albumin is usually small.

Renal albuminuria refers to albumin which has passed from the blood into the urine through the walls of the kidney tubules or the glomeruli.

Albuminuria sufficient to be readily recognized by the ordinary clinical methods probably never occurs as a physiologic condition, the so-called *physiologic albuminuria* appearing only under conditions which must be regarded as abnormal. Among these may be mentioned excessive muscular exertion in those unaccustomed to it; excessive ingestion of proteins (dietetic albuminuria); prolonged cold baths; the latter stages of pregnancy, and childbirth. In these conditions the albuminuria is ordinarily slight and transient. Albumin is frequently present in the urine of infants, possibly because the young kidney is particularly sensitive to irritants.

There are certain other forms of albuminuria which have still less claim to be called physiologic, but which are not always regarded as pathologic. Among these is *cyclic, orthostatic,* or *postural albuminuria.* This appears at certain periods of the day, which may vary in different cases, and disappears with rest in bed. Most frequently the maximum output of albumin occurs late in the afternoon. This form of albuminuria occurs, for the most part, in neurasthenic subjects during adolescence and is by no means rare. In some cases at least it is associated with curvature of the spine (lordotic albuminuria). The phenolsulphonephthalein test usually shows normal excretion. Tube casts may or may not be present. A considerable amount of protein precipitable by acetic acid in the cold has been noted in many cases. It is noteworthy in this connection that nephritis sometimes begins with a cyclic albuminuria.

In pathologic conditions, and in most, at least, of the "functional" conditions just enumerated, renal albuminuria may be referred to one or more of the following causes. In nearly all cases it is accompanied by tube-casts.

(*a*) *Circulatory changes in the kidney,* either anemia or congestion, as in excessive exercise, chronic heart disease, severe general anemia, and pressure upon the renal veins, as in the later stages of pregnancy. The quantity of albumin is usually but not always small. Its presence is constant or temporary, according to the cause. Most of the causes, if continued, will lead to degenerative changes in the kidney and even to nephritis.

(*b*) *Irritation of the Kidneys.*—Here there is slight damage to glomeruli or renal cells, with cloudy swelling or even more serious degeneration, but without definite nephritis. The amount of albumin is generally small and the condition transitory. This is probably the chief factor in toxic and febrile albuminuria, and it is at least a contributing factor in the albuminuria of pregnancy. Among drugs which in toxic doses may cause albuminuria are mercury, cantharides, turpentine, mustard, arsenic, lead, ether, etc. In febrile conditions, particularly in the acute infectious diseases, small or moderate amounts of albumin are frequently found in the urine, owing chiefly to the irritant effect of bacterial toxins. This is especially true of diphtheria, scarlet fever, pneumonia, typhoid fever, and acute streptococcic infections, in any of which the renal

condition may develop into true nephritis, with coincident increase in the amount of albumin.

(*c*) *Organic Changes in the Kidney.*—These include the inflammatory and degenerative changes commonly grouped together under the name of nephritis, and also renal tuberculosis and neoplasms. The amount of albumin eliminated in these conditions varies from minute traces to 20 gm., or even more, in the twenty-four hours, and, except in acute processes, bears little relation to the severity of the disease. In acute and chronic parenchymatous nephritis and in syphilis of the kidney the quantity is usually very large, from 1 to 2 per cent or more. In chronic interstitial nephritis it is small—frequently no more than a slight trace. It is variable in renal tuberculosis and neoplasms. In amyloid disease of the kidney the quantity is usually small, and serum-globulin may be present in especially large proportion or even alone. Roughly distinctive of serum-globulin is the appearance of an opalescent cloud when a few drops of the urine are dropped into a glass of distilled water.

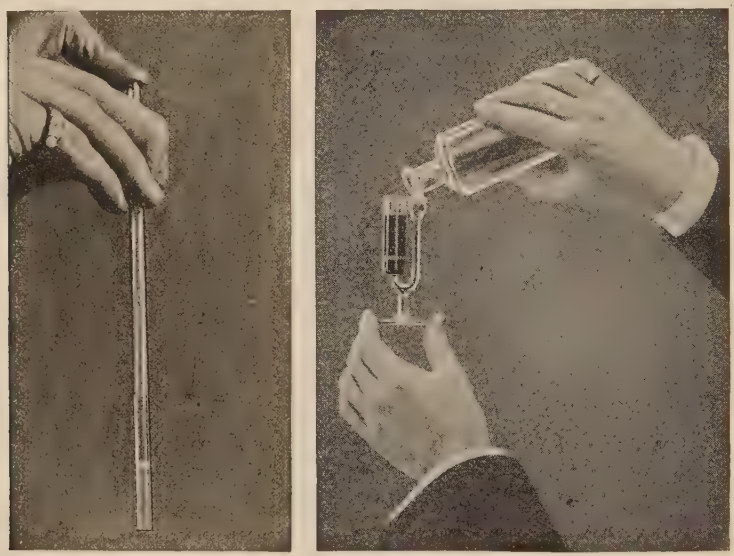
Detection of albumin depends upon its precipitation by chemicals or coagulation by heat. There are many tests, but none is entirely satisfactory, because other substances as well as albumin are precipitated. The most common source of error is mucin. When any considerable amount of mucin is present it can be removed by acidifying with acetic acid and filtering. Urine voided early in the evening or a few hours after a meal is most likely to contain albumin.

It is very important that urine to be tested for albumin be rendered clear by filtration or centrifugation. This is too often neglected in routine work. When ordinary methods do not suffice, it can usually be cleared by shaking up with a little purified talc, infusorial earth, or animal charcoal, and filtering. This will remove a part of the albumin by absorption, but the remainder is more easily detected. If the urine be alkaline, sufficient acetic acid should be added to make it acid to litmus. Vaughan has recently called attention to the fact that if bacteria be abundant in an alkaline urine, some of the bacterial proteins may go into solution and give the tests for albumin. In extremely concentrated urine certain of the urinary salts may interfere with the test for albumin. In such cases dilution of the urine will render the test more definite, even though the concentration of albumin is thereby reduced.

Albuminous urine foams markedly on shaking and the foam remains a long time. This gives a rough indication of the presence of albumin before the tests are made.

Technic of Ring or Contact Tests.—Since this simple and widely useful method of testing is best known in connection with the detection of albumin a general description is given at this place:

Take a few cubic centimeters of the heavier fluid in a conical test glass, hold the glass in an inclined position, and run the lighter fluid gently down the inside of the glass by means of a medicine-dropper so



A B

FIG. 46.—Showing two methods of performing the contact test for albumin: A, Boston's method, which is convenient for routine testing of a large number of urines, but is less accurate than the use of a conical test-glass as recommended in the text (Boston). B, Horismascope, adding the reagent. The white ring at the zone of contact is seen in the wider arm. This is an extremely satisfactory method for the office laboratory.

that it will form a layer on top of the other without mixing. In the case of the urine, which must be filtered before testing, it may be run in directly from the stem of the funnel by touching this against the wall of the test glass. If the test be positive, a sharply defined white or colored ring will appear where the two fluids come into contact. According to its color the ring is seen most clearly if viewed against a white or a black background, as the case may be; and one side of the test glass may be painted half white, half black, for this purpose. It is better, however, to use clear glass and to have the white or black background at a distance of some feet.

In our experience this is the most satisfactory technic. The common practice of taking the reagent in a narrow test-tube and pouring the urine in on top of it from a bottle is much inferior. Boston brings the fluids into contact in a glass pipet, which is immersed first in the lighter fluid and then (after wiping the outside of the pipet) in the heavier (Fig. 46). This is convenient for the routine testing of a large number of urines, but cannot be recommended for accuracy, owing to the small diameter of the column of fluid. Substitution of a medicine-dropper in place of the pipet renders Boston's method more convenient, but no more accurate. For those who do only a little testing the "horismascope" (Fig. 46) will be found very convenient and satisfactory. The instrument is, however, fragile and somewhat expensive.

The albumin tests here given are widely used and can be recommended for clinical purposes. They make no distinction between serum-albumin and serum-globulin. As a rule, the most sensitive tests are not the most useful for clinical purposes. The writers prefer Purdy's heat test and Roberts' ring test for routine testing, or Exton's method for a more sensitive test. The extremely sensitive trichloracetic acid test is used only in special cases.

1. **Trichloracetic Acid Test.**—The reagent consists of a saturated aqueous solution of trichloracetic acid to which magnesium sulphate is added to saturation. A simple saturated solution of the acid may be used, but addition of magnesium sulphate favors precipitation of globulin, and, by raising the specific gravity, makes the test easier to apply.

The test is carried out by the "ring" or "contact" method just described. If albumin be present a white, cloudy ring will appear where the two fluids come in contact.

This is an extremely sensitive test, but, unfortunately, both mucin and proteoses respond to it; urates, when abundant, may give a confusing white ring, and the reagent is comparatively expensive. It is not much used in routine work except as a control to the less sensitive tests.

2. **Sulphosalicylic acid** in 20 per cent. aqueous solution may be used in the same way as the trichloracetic acid reagent. It is fully as sensitive and is somewhat more reliable, in that urates and resins are not precipitated. This may also be applied by adding a few drops of the reagent to a few cubic centimeters of the urine in a test-tube and obtaining a white cloud in the presence of albumin, or by adding a bit of the sulphosalicylic acid in the solid state. The last is especially convenient for the practitioner who wishes to make albumin tests at the bedside.

Exton's method is an application of this test and is very satisfactory. To make the reagent dissolve 200 gm. sodium sulphate in about 750 c.c. of water with the aid of heat, cool, add 50 gm. sulphosalicylic acid, and dilute with water to 1000 c.c. The test is performed by mixing equal parts of the urine and reagent in a test-tube and warming gently. Boiling is not necessary or desirable. A white cloud shows the presence of albumin or globulin. The reaction is read while the fluid is warm, since secondary proteoses will cause a clouding when it cools. The Bence-Jones protein causes a heavy precipitate which clears partially or wholly upon boiling. Exton reports that the test is slightly less sensitive than Purdy's heat test.

3. **Roberts' Test.**—The reagent consists of pure nitric acid 1 part, and saturated aqueous solution of magnesium sulphate 5 parts. It is applied by the "ring" or "contact" method above described.

Albumin gives a white ring, which varies in density with the amount present, and when traces only are present may not appear for two or three minutes. A similar white ring may be produced by Bence-Jones' protein, primary proteose, thymol, and resinous drugs. White rings or cloudiness in the urine *above* the zone of contact may result from excess of urates or mucus. Colored rings near the junction of the fluids may be produced by iodids, urinary pigments, bile, or indican, but these are not so frequent as with Heller's test.

Roberts' test is one of the best for routine work, although the various rings are apt to be confusing to the inexperienced. It is a little more sensitive than the widely used **Heller's test,** which is performed in exactly the same way, using pure concentrated nitric acid; and it has the additional advantage that the reagent is not so corrosive. If in Heller's test the ring appears only after two minutes the amount of albumin is less than 0.0005 per cent.

4. **Purdy's Heat Test.**—Take a test-tube two-thirds full of urine, add about one-sixth its volume of saturated solution of sodium chlorid, and 5 to 10 drops of 50 per cent. acetic acid. Mix, and boil the upper inch, holding the tube with the fingers near the bottom. A white cloud in the heated portion shows the presence of albumin. A faint cloud is best seen when viewed against a black background at a distance of 2 or 3 feet.

This is a valuable test for routine work. It is simple, sufficiently accurate for clinical purposes, and has practically no fallacies. *Addition of the salt solution by raising the specific gravity prevents precipitation of mucin.* Bence-Jones' protein may produce a white cloud, which disappears upon boiling and reappears upon cooling.

5. **Heat and Nitric Acid Test.**—This is one of the oldest of the

albumin tests, and, if properly carried out, one of the best. Boil about 5 c.c. of filtered urine in a test-tube and add 1 to 3 drops of concentrated nitric acid. The tube may be held with a test-tube clamp, or simply with a strip of muslin or filter-paper, the center of which is folded once around the neck of the tube. A white cloud or flocculent precipitate (which usually appears during the boiling, but if the quantity be very small only after addition of the acid) denotes the presence of albumin. A similar white precipitate, which disappears upon addition of the acid, is due to earthy phosphates. The acid should not be added before boiling, and the proper amount should always be used; otherwise, part of the albumin may fail to be precipitated or may be transformed to acid-albumin and redissolved. Resinous drugs might give a white cloud with this test, but this will disappear upon addition of alcohol. A white cloud which appears only after cooling may be due to Bence-Jones' protein or to primary proteose. Effervescence upon addition of the acid is generally due to carbonates from the food, notably lemonade.

A decided advantage of this test is the fact that it allows a rough estimation of the amount of albumin from the volume of the sediment after standing over night. When the entire fluid solidifies the albumin amounts to 2 to 3 per cent. Sediments reaching to one-half, one-third, one-fourth, and one-tenth the height of the column of urine correspond respectively to about 1, 0.5, 0.25, and 0.1 per cent. albumin. When there is only a slight cloudiness the albumin does not exceed 0.01 per cent.

Scheme for Recording Results of Tests.—It is customary to record qualitative albumin tests in such a way as to convey a rough idea of the quantity of albumin. The plan is very useful clinically, but it must be remembered that other factors than the quantity of albumin—notably the salt content of the urine—affect the amount and character of the clouding or precipitate. Also the interpretation of the terms used will vary greatly with different persons. The following scheme is widely used. It is given both for the heat tests, such as heat and nitric acid, and for contact tests, such as Heller's.

1. *Trace.*—The cloudiness or ring can just be seen, usually best against a black background.
2. *Small Amount.*—Heat test: Cloud is distinct and granular without definite flocculles. Precipitate settles to about one-tenth height of column of urine in twenty-four hours. Contact test: The ring is dense, but not wholly opaque when viewed from above. Represents about 0.1 per cent. of albumin.
3. *Moderate Amount.*—Heat test: Cloud is dense with very marked flocculation. Contact test: The ring is heavy, wholly opaque, sometimes curdy. Represents about 0.2 to 0.3 per cent. of albumin.

4. *Large Amount.*—Heat test: Precipitate is very heavy and curdy and may become a solid mass. Contact test: The ring is very dense. Represents more than 0.5 per cent. albumin.

The result can be recorded "Albumin 1, 2, 3, or 4," according to the estimated amount of albumin.

Quantitative Estimation.—Accurate estimation of albumin is seldom necessary in routine clinical work. Ordinarily the information obtainable from properly conducted qualitative tests will suffice. When more definite figures are required, the following simple methods are available. Of these, the last, Exton's new method, is by far the most accurate.

FIG. 47.—Esbach's albuminometer, improved form.

1. **Esbach's Method.**—The urine must be clear, of acid reaction, and not concentrated. Always filter before testing, and, if necessary, add acetic acid and dilute with water, making allowance for the dilution in the final calculation. Esbach's tube (Fig. 47) is essentially a test-tube with a mark U near the middle, a mark R near the top, and graduations $\frac{1}{2}$, 1, 2, 3, etc., near the bottom. Fill the tube to the mark U with urine and to the mark R with the reagent. Close with a rubber stopper, invert slowly several times, and set aside in a cool place. At the end of twenty-four hours read off the height of the precipitate. This gives the amount of albumin in *grams per liter, and must be divided by 10 to obtain the percentage.*

Lenk advises addition of a small quantity of powdered charcoal, pumice, or kaolin after adding Esbach's reagent. This hastens sedimentation, which is complete in ten minutes to half an hour. Andresen uses 0.1 to 0.2 gm. of barium sulphate.

Esbach's reagent consists of picric acid, 1 gm.; citric acid, 2 gm., and distilled water, to make 100 c.c.

2. **Tsuchiya's Method.**—This is carried out in the same manner as the Esbach method, using the following reagent:

Phosphotungstic acid	1.5 gm.
Alcohol (96 per cent.)	95.0 c.c.
Concentrated hydrochloric acid	5.0 "

The urine should be diluted to a specific gravity not exceeding 1.008. The method is said to be much more accurate than the original Esbach method, particularly with small quantities of albumin.

3. **Purdy's Centrifugal Method.**—In a graduated centrifuge tube take 10 c.c. of urine, 2 c.c. of 50 per cent. acetic acid, and 3 c.c. of 10 per cent. solution of potassium ferrocyanid. Mix and let stand ten minutes. Centrifugalize at 1500 revolutions per minute for three minutes or until the bulk of the precipitate remains constant. Read off the volume of precipitate and find the corresponding percentage of albumin by reference to the following table. When the amount of albumin is excessive, dilute the urine until the volume of precipitate falls below 1.5 c.c. and multiply the result by the dilution employed.

PURDY'S QUANTITATIVE METHOD FOR ALBUMIN

Table showing the relation between the volume of precipitate after centrifugation and the gravimetric percentage of albumin.

Volume of of precipitate, c.c.	Percentage by weight of dry albumen.	Volume of precipitate, c.c.	Percentage by weight of dry albumin.	Volume of precipitate, c.c.	Percentage by weight of dry albumin.
0.10	0.021	1.1	0.229	2.1	0.438
0.20	0.042	1.2	0.25	2.2	0.458
0.30	0.063	1.3	0.271	2.3	0.479
0.40	0.083	1.4	0.292	2.4	0.5
0.50	0.104	1.5	0.313	2.5	0.521
0.60	0.125	1.6	0.333	2.6	0.542
0.70	0.146	1.7	0.354	2.7	0.563
0.80	0.167	1.8	0.375	2.8	0.583
0.90	0.187	1.9	0.396	2.9	0.604
1.00	0.208	2.0	0.417	3.0	0.625

4. **Exton's Quantitative Method.**—This uses a sulphosalicylic acid reagent. It is turbidimetric, and the quantity of albumin is read directly by comparison with a set of standard tubes representing, 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mg. protein for each 100 c.c. It may be necessary to dilute urine containing a large amount of albumin so that a comparison can be made with the standard tubes. All tubes must be scrupulously clean.

Add 2 or 3 c.c. of reagent[1] to an equal amount of urine and allow to stand for at least five minutes. Warm the urine by passing the tube slowly through the flame several times, but do not boil. Make all suspensions homogeneous in the standard tubes by gently inverting. The

[1] *Formula of Reagent:* Dissolve 50 gm. of sulphosalicylic acid and 10 gm. of sodium sulphate in 800 c.c. distilled water; add 25 c.c. of 0.40 per cent. aqueous solution of bromphenol blue. Make up to 1 liter and filter through acid-washed paper.

comparisons are best made in the apparatus, and with the standard tubes, furnished by the manufacturer.[1]

A somewhat similar method now used by the Metropolitan Life Insurance Company has been devised by Kingsbury and his assistants.[2] They use as a reagent 3 per cent. sulphosalicylic acid, adding 7.5 c.c. to 2.5 c.c. of urine. The artificial quantitative standards are made from dilutions of *formazin* held in suspension in gelatin, and are for sale by the Fales Chemical Company, New York, N. Y.

(2) **Mucin.**—Traces of the substances (mucin, mucoid, nucleoprotein, and so forth) which are loosely classed under this name are present in normal urine; increased amounts are observed in irritations and inflammations of the mucous membrane of the urinary tract or the vagina. They are of interest chiefly because they may be mistaken for albumin in most of the tests. If the urine be diluted with water and acidified with acetic acid without heating, the appearance of a white cloud indicates the presence of mucin.

Mucin and mucoid are glycoproteins, and upon boiling with an acid or alkali, as in Fehling's test, yield a carbohydrate substance which reduces copper.

(3) **Bence-Jones' Protein.**—The protein known by this name was originally classed as an albumose, but its protein nature is now well established. It was formerly regarded as practically pathognomonic of multiple myeloma, but has recently been found in a number of cases of chronic leukemia, of both lymphatic and myelogenous types, in osteomalacia, and, along with albumin, in some cases of chronic nephritis with high blood-pressure and edema, and also in certain seemingly healthy young persons with high blood-pressure (Miller and Baetjer).

To detect Bence-Jones' protein the urine is slightly acidified with acetic acid and gently heated in a water-bath. If this substance be present, the urine will begin to be turbid at about 40° C. and a precipitate will form at about 60° C. As the boiling-point is reached the precipitate wholly or partially dissolves. It reappears upon cooling. It may easily be overlooked in the presence of albumin. If the urine be

[1] Lehn and Fink, Inc., New York, N. Y., who also furnish the reagent.

[2] Kingsbury, F. B., Clark, C. P., Williams, Gertrude, and Post, Anna L.: The rapid determination of albumin in urine, Jour. Lab. and Clin. Med., 1926, xi, 981–989.

filtered while at or near the boiling temperature, the albumin may be removed, leaving the Bence-Jones' protein.

As a confirmatory test the protein may be precipitated by adding nitric acid at room temperature. This precipitate wholly or partially clears up on boiling and reappears on cooling. The protein may also be precipitated by alcohol, and if the precipitate be collected *at once* by centrifugation it readily dissolves in water. For complete identification of the protein the reader is referred to the more comprehensive works upon biochemistry.

(4) **Proteoses.**—These are intermediate products in the digestion of proteins and are frequently, although incorrectly, called albumoses. Two groups are generally recognized: *primary proteoses*, which are precipitated upon half-saturation of their solutions with ammonium sulphate; and *secondary proteoses*, which are precipitated only upon complete saturation.

The proteoses appear in the urine whenever a considerable amount of tissue or exudate is being autolyzed and absorbed, as in febrile and malignant diseases and chronic suppurations, during resolution of pneumonia, and in many other conditions, but their clinical significance is indefinite. In pregnancy, albumosuria may be due to absorption of amniotic fluid and, later, to involution of the puerperal uterus.

The proteoses are not coagulable by heat, but are precipitated by such substances as trichloracetic acid, sulphosalicylic acid, and phosphotungstic acid. The primary proteoses alone are precipitated by adding concentrated nitric acid. This precipitate disappears on heating and reappears on cooling.

Proteoses may be detected by acidifying the urine with acetic acid, boiling, filtering while hot to remove mucin, albumin, and globulin, and testing the filtrate by the trichloracetic acid test. As above indicated, the nitric acid test, and half and complete saturation with ammonium sulphate, will separate the two groups.

2. Sugars.—Various sugars may at times be found in the urine. Dextrose is by far the most common, and is the only one of much clinical importance. Levulose, lactose, and some others are occasionally met.

(1) **Dextrose** (**Glucose**).—Traces of glucose and other sugars too small to respond to the ordinary tests are present in the urine in

health. The presence of glucose in appreciable amount constitutes *glycosuria* or *glycuresis*, and is the result of increase of dextrose in the blood (hyperglycemia), or of lowered renal threshold for sugar, or both. These are discussed on page 345.

Transitory glycosuria is unimportant, is generally slight, and may occur in many conditions, as after general anesthesia and administration of certain drugs, in some cases of hyperthyroidism, in pregnancy, and following shock and head injuries. Recently attention has been directed to glycosuria following strong emotions (anger, fear, anxiety), due, according to Cannon, to increased adrenal secretion leading to sudden mobilization of dextrose which had been stored as glycogen. The urine of a considerable percentage of a class of students will give positive tests for sugar following a long and hard examination. The possibility that a trace of sugar found in a patient's urine after a physical examination may be due to his anxiety must be kept in mind. Glycosuria may also occur after eating excessive amounts of carbohydrates (alimentary glycosuria). The "assimilation limit" varies with different individuals and with different conditions of exercise. It also depends upon the kind of carbohydrate. The normal for pure dextrose is generally given as about 100 to 200 gm., but more recent work has shown that in many individuals glycuresis cannot be induced by much larger amounts even up to the maximum (400 to 500 gm.) which the stomach will tolerate. Glycuresis following ingestion of 100 gm. or less is definitely abnormal, indicating lowered renal threshold diminished capacity of the liver to store glucose as glycogen, or disturbance of carbohydrate metabolism. Excretion lasts for a period of four or five hours.

Persistent, although not necessarily continuous, glycosuria has been noted in brain injuries involving the floor of the fourth ventricle and in renal glycosuria (p. 217), of which it is the essential symptom. As a rule, however, persistent glycosuria is diagnostic of diabetes mellitus, and this is by far its most important indication. The amount of glucose eliminated in diabetes is usually considerable, and is sometimes very large, reaching 500 grams, or even more, in twenty-four hours, but it does not bear any uniform relation to the severity of the disease. Glucose may, on the other hand, be almost or entirely absent as a result of careful restriction of the diet, and in mild cases it may appear only about two or three hours

after ingestion of considerable quantities of carbohydrate. Diabetes is discussed more fully on page 216.

Detection of Dextrose.—Albumin if present in any considerable quantity interferes with precipitation of copper in the copper tests, and should be removed by acidifying with acetic acid, boiling, and filtering.

1. **Haines's Test.**—Take about 4 c.c. of Haines's solution in a test-tube, boil, examine carefully for a precipitate, and, if none is present, add 6 or 8 drops of urine while keeping the reagent hot but not boiling. A yellow or red precipitate, which settles readily to the bottom, shows the presence of sugar. Neither precipitation of phosphates, as a light, flocculent sediment, nor simple decolorization of the reagent should be mistaken for a positive reaction. The test will detect about 0.1 per cent. of dextrose.

This is one of the best of the copper tests, all of which depend upon the fact that in strongly alkaline solutions glucose reduces cupric hydrate to cuprous hydrate (yellow) or cuprous oxid (red). They are somewhat inaccurate because they make no distinction between glucose and less common forms of sugar; because certain normal substances when present in excess, especially mucin, uric acid, and creatinin, may reduce copper, and because many drugs—for example, chloral, chloroform, copaiba, acetanilid, benzoic acid, morphin, sulphonal, salicylates, aspirin—are eliminated as copper-reducing substances. To minimize these fallacies *dilute the urine, if it be concentrated; do not add more than the specified amount of urine, and do not boil after the urine is added.* If chloroform has been used as a preservative, it should be removed by boiling the urine before making the test.

Haines's solution is prepared as follows: Completely dissolve 2 gm. pure copper sulphate in 16 c.c. distilled water, and add 16 c.c. pure glycerin; mix thoroughly, and add 156 c.c. of 5 per cent. potassium hydroxid. The solution keeps well.

2. **Fehling's Test.**—Two solutions are required—one containing 34.64 gm. pure crystalline copper sulphate in 500 c.c. distilled water; the other, 173 gm. Rochelle salt and 100 gm. potassium hydroxid in 500 c.c. distilled water. Mix equal parts of the two solutions in a test-tube, dilute with 3 or 4 volumes of water, and boil. Add the urine a little at a time, heating, but not boiling, between additions. In the presence of dextrose a heavy red or yellow precipitate will appear. The quantity of urine should not exceed that of the reagent. The fallacies mentioned under Haines's test apply equally to this.

3. **Benedict's Test.**—This test promises to displace all other reduc-

tion tests for sugar. The reagent will detect 0.015 to 0.02 per cent. of dextrose and is thus several times as sensitive as Haines's or Fehling's solutions. It is not reduced by uric acid, creatinin, chloroform, or the aldehyds. It consists of:

Copper sulphate (pure crystallized)	17.3	gm.
Sodium or potassium citrate	173.0	"
Sodium carbonate (crystallized) (or 100 gm. of anhydrous sodium carbonate).	200.0	"
Distilled water, to make	1000.0	c.c.

Dissolve the citrate and carbonate in 700 c.c. of water, with the aid of heat, and filter. Dissolve the copper in 100 c.c. of water and pour slowly into the first solution, stirring constantly. Cool, and make up to 1 liter. The reagent keeps indefinitely. *It cannot be used for quantitative estimations.*

Take about 5 c.c. of this reagent in a test-tube, heat to boiling to make sure that none of the copper is precipitated by heat alone, and add *8 or 10 drops* (*not more*) of the urine. Heat to vigorous boiling, keep at this temperature for one or two minutes, and allow to cool slowly. In the presence of glucose the entire body of the solution will be filled with a precipitate, which may be red, yellow, or green in color. When traces only of glucose are present, less than 0.1 per cent., the precipitate may appear only upon cooling. In the absence of glucose the solution remains clear or shows only a faint *bluish* precipitate, due to urates. The long boiling is inconvenient, especially when there is much bumping in the tube. It will therefore generally be found more satisfactory, particularly when a large number of urines must be tested, to place the tubes in a beaker of water which is kept at the boiling-point for five minutes.

4. **Phenylhydrazin Test.**—*Kowarsky's Method.*—The following directions include certain modifications which have been worked out by C. S. Bluemel at Boulder, Colorado: In a wide test-tube take 5 drops pure phenylhydrazin, 10 drops glacial acetic acid, and 1 c.c. saturated solution of sodium chlorid. A curdy mass results. Add 3 or 4 c.c. of the urine and 4 or 5 c.c. of water. Boil vigorously for two or three minutes. The annoying bumping can be reduced or obviated by shaking continually or, much better, by placing in the test-tube a number of pieces of glass tubing, varying in length from $1\frac{1}{2}$ to 3 inches, so as to produce an organ-pipe effect. The volume of fluid remaining after boiling should be 2 to 3 c.c. Set aside to cool or, if the glass tubes were used, pour the fluid into another hot test-tube and allow to cool. Examine the sediment with the microscope, using a 16-mm. objective. If

dextrose be present, characteristic crystals of phenylglucosazone will be seen. These are yellow, needle-like crystals arranged mostly in clusters or in sheaves (Fig. 48). When traces only of glucose are present the crystals may not appear for one-half hour or more. The best crystals are obtained when the fluid is cooled very slowly and is not agitated during cooling. The test-tubes and pieces of tubing can be cleaned when necessary by boiling in a solution of caustic soda or acetic acid.

This is an excellent test for clinical work. Bluemel finds that when applied as above directed, with the tubing to prevent bumping, it will readily detect 0.025 per cent. of dextrose in urine, the crystals appearing in three to four hours. The test has practically no fallacies except levulose, which is a fallacy for all the ordinary tests. Other carbohydrates which are capable of forming crystals with phenylhydrazin are

FIG. 48.—Crystals of phenylglucosazone (yellow) from diabetic urine—Kowarsky's test (× 500)

extremely unlikely to do so when the test is applied directly to the urine. Even if not used routinely, this test should always be resorted to when the copper tests give a positive reaction in doubtful cases.

5. **Fermentation Test.**—This is simple and reliable, but owing to the time required it is not much used in routine work, except as an aid in distinguishing dextrose from other forms of sugar. It is carried out in the same manner as the quantitative test (p. 148). Improvised devices which answer well for the purpose are shown in Figure 49. If the fermenting urine be placed in the incubator at 40° to 45° C. the result should be definite within an hour or two; and it can be still further hastened by increasing the amount of yeast.

Scheme for Recording Results of Tests.—Unless a quantitative estimation is made sugar is generally reported simply as "present" or

"absent." If it be desired, when recording a positive qualitative test, to convey some notion of the quantity of sugar present, the following scheme is suggested when Benedict's solution is employed:

1. S. T., *slight trace.* No reduction is evident during two minutes' boiling with 8 or 10 drops of urine, but it appears upon cooling.
2. T., *trace.* With 8 drops of urine the reaction occurs after about one minute's boiling.
3. M. A., *moderate amount.* With 8 or 10 drops of urine the reaction occurs after ten or fifteen seconds' boiling.
4. L. A., *large amount.* Reduction occurs almost immediately after adding 2 drops of urine to the boiling reagent.

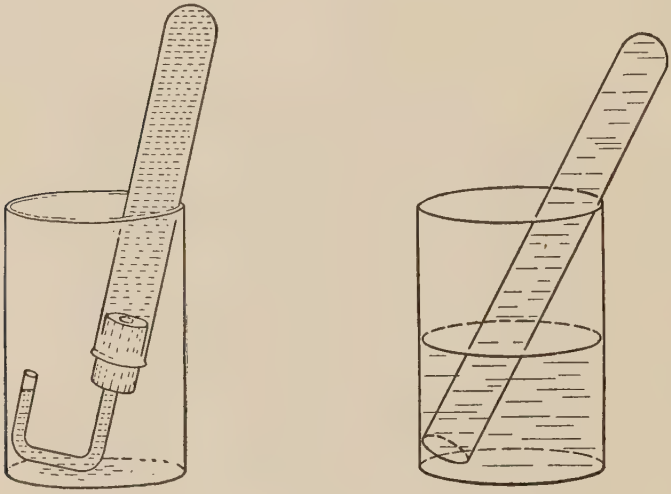


FIG. 49.—Simple devices for fermentation test for dextrose.

Owing to the many variable factors, it is impossible to set down with any degree of accuracy the percentages of dextrose covered by the four terms.

Quantitative Estimation.—In quantitative work Fehling's solution, for so many years the standard, has been largely displaced by Benedict's quantitative solution, which appears to be more exact and more satisfactory than any other titration method available for sugar work. The older method is still preferred by some and both are therefore given.

Should the urine contain much glucose, it must be diluted before making any quantitative test, allowance being made for the dilution in the subsequent calculation. Albumin, if present, must

be removed by acidifying a considerable quantity of urine with acetic acid, boiling, and filtering. Any water lost during the boiling should be replaced before filtering.

A rough but sometimes useful approximation of the amount of sugar in the urine of a diabetic patient can be made by estimating the total solids (p. 93), subtracting what may be regarded as normal for the individual, and regarding the remainder as sugar.

1. **Fehling's Method.**—Take 10 c.c. of Fehling's solution (made by mixing 5 c.c. each of the copper and alkaline solutions described on page 143) in a flask or beaker, add 3 or 4 volumes of water, boil, and add the urine very slowly from a buret until the solution is completely decolorized, heating but not boiling after each addition.

Fehling's solution is of such strength that the copper in 10 c.c. will be reduced by exactly 0.05 gm. of dextrose. Therefore, the amount of urine required to decolorize the test solution contains just 0.05 gm. dextrose, and the percentage is easily calculated.

The chief objection to Fehling's method is the difficulty of determining the end-point. The use of an "outside indicator," however, obviates this. When reduction is thought to be complete, a few drops of the solution are filtered through a fine-grained filter-paper on to a porcelain plate, quickly acidified with acetic acid, and mixed with a drop of 10 per cent. potassium ferrocyanid. Immediate appearance of a reddish-brown color shows the presence of unreduced copper.

A somewhat simpler application of this method, which is accurate enough for most clinical purposes, is as follows: Take 1 c.c. of Fehling's solution in a large test-tube, dilute with about 5 c.c. of water, heat to boiling, and, while keeping the solution hot but not boiling, add the urine, drop by drop, from a medicine-dropper until the blue color is entirely gone. Toward the end add the drops very slowly, not more than 4 or 5 a minute. Divide 10 by the number of drops required to discharge the blue color; the quotient will be the percentage of glucose. If 20 drops were required, the percentage would be $10 \div 20 = 0.5$ per cent. It is imperative that the drops be of such size that 20 of them will make 1 c.c. Test the dropper with urine, not water, and hold it always at the angle which will give the right sized drop. If the drops are too large, draw out the tip of the dropper; if too small, cut off the tip. The method is, of course, more reliable if an accurate 1-c.c. pipet, graduated in tenths, be used. In this case the calculation consists in dividing 5 by the number of tenths of a cubic centimeter of urine required.

2. **Benedict's Method.**—The following modification of his copper solution has been offered by Benedict for quantitative estimations.

The reagent consists of:

Copper sulphate (pure crystallized)	18.0 gm.
Sodium carbonate (crystallized)	200.0 "
(or 100 gm. of anhydrous sodium carbonate).	
Sodium or potassium citrate, C. P.	200.0 "
Potassium sulphocyanate, C. P.	125.0 "
Potassium ferrocyanid solution (5 per cent.)	5.0 c.c.
Distilled water, to make	1000.0 "

With the aid of heat dissolve the carbonate, citrate, and sulphocyanate in about 700 c.c. of the water and filter. Dissolve the copper in 100 c.c. of water and pour slowly into the other fluid, stirring constantly. Add the ferrocyanid solution, cool, and dilute to 1000 c.c. Only the copper need be accurately weighed. This solution is of such strength that 25 c.c. are reduced by 0.05 gm. glucose. It keeps well.

To make a sugar estimation take 25 c.c. of the reagent in a small flask, add 10 to 20 gm. of sodium carbonate crystals (or one-half this weight of anhydrous sodium carbonate) and a small quantity of powdered pumice-stone or talcum. Heat to boiling, and add the urine a little at a time, but fairly rapidly, from a buret until a chalk-white precipitate forms and the blue color of the reagent begins to fade. After this point is reached, add the urine a drop at a time until the last trace of blue just disappears. This end-point is easily recognized. During the whole of the titration the mixture must be kept vigorously boiling. Loss by evaporation must be made up by adding water. Note the quantity of urine required to discharge the blue color: this contains exactly 0.05 gm. dextrose, and the percentage is easily calculated.

Benedict's solution may be employed in the simplified method given for Fehling's solution on page 147 by using 2.5 c.c. of Benedict's quantitative solution, without addition of water, 1 gm. of anhydrous sodium carbonate, and a small piece of cotton to preventing bumping during the boiling.

3. **Fermentation Method.**—This is convenient and satisfactory, its chief disadvantage being the time required. It depends upon the fact that glucose is fermented by yeast with evolution of CO_2. The amount of gas evolved is an index of the amount of glucose. No preservative must have been added to the urine. Einhorn's saccharimeter (Fig. 50) is the simplest apparatus available; Lohnstein's is probably the most accurate.

The urine must be so diluted as to contain not more than 1 per cent. of glucose. A fragment of fresh yeast-cake about the size of a pea is mixed with a definite quantity of the urine measured in the tube which accompanies the apparatus. The exact amounts of yeast and urine are

unimportant. It should form an emulsion free from lumps or air-bubbles. A pinch of tartaric acid may be added to prevent bacterial fermentation. The long arm of the apparatus and about half the bulb are then filled with the mixture, all bubbles being carefully discharged by tipping the instrument with the thumb over the opening, and the instrument is stood in a warm place, preferably an incubator. At the end of fifteen to twenty-four hours at room temperature, or about three hours in an incubator, fermentation will be complete, and the percentage of glucose can be read off upon the side of the tube. The result must then be multiplied by the degree of dilution. Since yeast itself sometimes gives off gas, a control test must be carried out with normal urine and the amount of gas evolved must be subtracted from that of the test. A control should also be made with a known glucose solution to make sure that the yeast is active. It has recently been shown that yeast can split off carbon dioxid from amino-acids, so that the results with the fermentation method may sometimes be a little high.

The method is not applicable to urine which is undergoing ammoniacal fermentation.

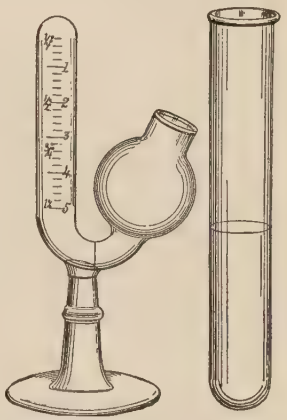


FIG. 50.—Einhorn's saccharimeter.

(2) **Levulose,** or **fruit sugar,** is seldom present in urine except in association with dextrose, and has about the same significance. According to von Noorden, its appearance in diabetes indicates an advanced case. Its name is derived from the fact that it rotates polarized light to the left.

Detection of Levulose.—Levulose responds to all the tests above given for dextrose. It may be distinguished from dextrose by the following:

Borchardt's Test.—Mix about 5 c.c. each of the urine and 25 per cent. hydrochloric acid (concentrated HCl, 2 parts; water, 1 part) in a test-tube and add a few crystals of resorcinol. Heat to boiling and boil for not more than one-half minute. In the presence of levulose a red color appears. Cool in running water, pour into a beaker, and render slightly alkaline with solid sodium or potassium hydroxid. Return to the test-tube, add 2 or 3 c.c. of acetic ether, and shake. If levulose be present, the ether will be colored yellow. A similar yellow color will follow administration of rhubarb and senna.

If indican be present the test must be modified as follows: Perform Obermayer's test and extract the indican with chloroform. Reduce the acidity of the indican-free urine by adding one-third its volume of water, add a few crystals of resorcinol, and proceed with Borchardt's test.

Quantitative Estimation of Levulose.—The methods are the same as for dextrose (p. 146); 25 c.c. of Benedict's quantitative solution are reduced by 0.053 gm. levulose.

(3) **Lactose,** or **milk-sugar,** is sometimes present in the urine of nursing women and in that of women who have recently miscarried. It is of interest chiefly because it may be mistaken for glucose. *It does not ferment yeast but reduces copper,* 0.0676 gm., being equivalent to 25 c.c. of Benedict's quantitative solution. In strong solution it can form crystals with phenylhydrazin, but is extremely unlikely to do so when the test is applied directly to the urine.

Rubner's Test.—To about 10 c.c. of the urine add 2 or 3 gm. of lead acetate, shake well, and filter into a test tube. Boil the filtrate, add 1 or 2 c.c. strong ammonia and heat again. If lactose be present, the solution turns brick-red and a red precipitate will separate. The precipitate is the criterion. Glucose gives a red solution with yellow precipitate.

(4) **Maltose** and **cane-sugar** are of little or no clinical importance. Maltose has been found along with dextrose in diabetes. It reduces copper, 0.074 gm. being equivalent to 25 c.c. of Benedict's solution. Cane-sugar (sucrose) is sometimes added to the urine by malingering patients. It does not reduce copper, and hence when it has been added passes unrecognized by the physician who uses the copper tests only. Both sugars are fermentable by yeast.

(5) **Pentoses.**—These sugars are so named because the molecule contains 5 atoms of carbon. Vegetable gums form their chief source. They reduce copper strongly but slowly, and give crystals with phenylhydrazin, but do not ferment with yeast.

Pentosuria is uncommon. It has been noted after ingestion of large quantities of pentose-rich substances, such as cherries, plums, and fruit juices, and is said to be fairly constant in habitual use of

morphin. It sometimes accompanies glycosuria in diabetes. An obscure chronic form of pentosuria without clinical symptoms has been observed most commonly in the Hebrew race. The pentose excreted in these cases is believed to be optically inactive arabinose, although recent studies indicate that ribose is present in some cases at least.

Bial's Orcinol Test.—Dextrose is first removed by fermentation. About 5 c.c. of Bial's reagent is heated in a test-tube, and after removing from the flame the urine is added drop by drop, not exceeding 20 drops in all. The appearance of a green color denotes pentose.

The reagent consists of:

Hydrochloric acid (30 per cent.)	500 c.c.
Ferric chlorid solution (10 per cent.)	25 drops
Orcinol	1 gm.

3. Acetone Bodies.—This is a group of closely related substances—acetone, diacetic acid, and beta-oxybutyric acid—whose chief source is in faulty catabolism of fats whereby the fatty acids fail to be completely oxidized. The underlying cause of this failure is not necessarily always the same, but is most frequently associated with defective carbohydrate metabolism. Normally, the metabolism of the carbohydrates furnishes the oxygen which is utilized for oxidation or "burning" of the fats of the body, hence it is said that "the fats burn in the flame of the carbohydrates." The amount of fat which can be completely oxidized bears a definite ratio to the amount of carbohydrate burned. When the supply of carbohydrates is deficient, as in starvation, or when their metabolism is defective, as in diabetes mellitus, there is insufficient oxygen available to properly burn the fats, and "the fire smokes." The result is an excessive production of the acetone bodies which are the products of this incomplete combustion. Formerly beta-oxybutyric acid was held to be the mother substance, but it is now believed that diacetic acid is first formed and that the others are derived from it.

To what extent these substances are toxic is unsettled, but it is quite clear that excessive production of diacetic and oxybutyric acids within the body leads, by virtue of their acid nature, to the condition known as acid intoxication. Since acetone bodies are ketones, this form of acidosis is sometimes given a special name—*ketosis*. The existence of this condition or, rather, the tendency

toward it, since there may be moderate acetonuria without definite acidosis, is shown by the presence of acetone bodies in the urine. When the condition is very mild, acetone occurs alone; as it grows more marked, diacetic acid and beta-oxybutyric acid are also found.

(1) **Acetone.**—Minute traces, too small for the ordinary tests, may be present in the urine under normal conditions. Larger amounts are not uncommon when the intake of carbohydrate is limited, and in fevers, gastro-intestinal disturbances, and certain nervous disorders. A notable degree of acetonuria has likewise been observed in cachectic conditions, in pernicious vomiting of pregnancy, in eclampsia, and in the serious and often fatal toxic condition which is now recognized as a not infrequent late effect of anesthesia, particularly of chloroform anesthesia. This postanesthetic toxemia seems to be more likely to appear and to be more severe when the urine contains any notable amount of acetone before operation, which suggests the importance of routine testing for acetone in surgical cases. It is not to be assumed that all of the toxic conditions enumerated are primarily acid intoxications nor that the symptoms are necessarily due to acidosis. It is more likely that the acetone bodies are formed secondarily as a result of the action of other toxic substances. Indeed, in many cases there may be little or no acidosis as measured by blood tests.

Acetonuria finds its chief clinical importance in connection with diabetes mellitus. It occurs intermittently in some mild cases, fairly regularly in most advanced cases, although much depends upon the diet, and is always present in severe cases. Tests for acetone are fully as important as tests for sugar in diabetes. A progressive increase—as measured by the strength of qualitative tests—is a grave prognostic sign, since acidosis due to the acetone bodies is probably the chief cause of the dreaded diabetic coma. Acetonuria can be diminished temporarily by more liberal allowance of carbohydrates in the diet.

Acetonuria from any cause is apt to be especially marked in children, and this doubtless plays an important part in acute and chronic diseases of childhood, especially in those requiring a restricted diet. In fact, the urine of a considerable percentage of young children shows acetone in appreciable quantities under normal conditions.

According to Folin, acetone is usually present in only small amounts in the above-mentioned conditions, the substance shown by the usual tests, particularly after distillation of the urine, being really diacetic acid. In this connection Frommer's test is to be recommended, since it does not require distillation, and does not react to diacetic acid unless too great heat is applied.

Owing to the marked and variable loss of acetone through the lungs a quantitative estimation is not of much value. In a case of diabetes, after the existence of an acidosis has been established by the detection of acetone bodies, it is better to rely upon an estimation of ammonia as a measure of its severity. The best measure of acidosis, applicable to all forms, is, however, the determination of the CO_2-combining power of the blood (p. 350).

FIG. 51.—A simple distilling apparatus. The longer the delivery tube, the better will the vapor condense. Condensation may also be facilitated by immersing the test-tube in a glass of cold water.

Detection of Acetone.—The urine may be tested directly, but it is much better to distil it after adding a little phosphoric or hydrochloric acid to prevent foaming, and to test the first few cubic centimeters of distillate. A simple distilling apparatus is shown in Figure 51. The test-tube may be attached to the delivery tube by means of a two-hole rubber cork as shown, the second hole serving as an air vent, or, what is much less satisfactory, it may be tied in place with a string. Should the vapor not condense well, the test-tube may be immersed in a glass of cold water. If a sufficiently long delivery tube be used (2 feet), there will be little difficulty about condensation.

When diacetic acid is present, a considerable proportion will be converted into acetone during distillation.

1. **Gunning's Test.**—To about 5 c.c. of urine or distillate in a test-tube add 5 drops of strong ammonia, and then Lugol's solution in sufficient quantity to produce a black cloud which does not immediately disappear. This cloud will gradually clear up and, if acetone be present, iodoform, usually crystalline, will separate out. The iodoform can be

recognized by its odor, especially upon heating (there is danger of explosion if the mixture be heated before the black cloud disappears), or by detection of the crystals microscopically. The latter, alone, is dependable, unless one has an acute sense of smell. The odor of iodin, which is also present, is often confusing. Iodoform crystals are yellowish six-pointed stars or six-sided plates (Fig. 52).

This modification of Lieben's test is less sensitive than the original, but is sufficient for all clinical work; it has the advantage that alcohol does not cause confusion, and especially that the sediment of iodoform is practically always crystalline. When it is applied directly to the urine, phosphates are precipitated and may form large, feathery, star-shaped crystals which are confusing to the inexperienced (Fig. 61). Albumin prevents formation of the iodoform crystals, and when it is present, the urine must be distilled for the test.

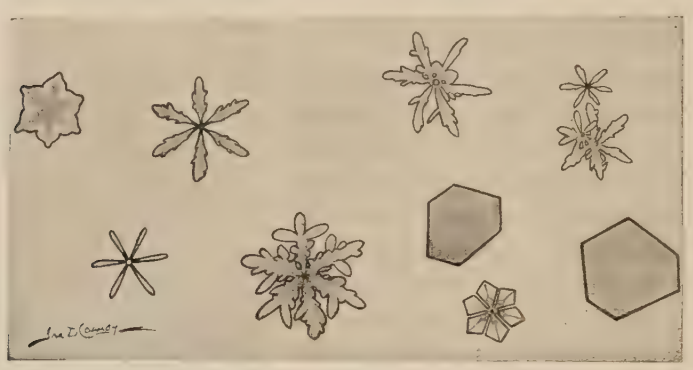


FIG. 52.—Iodoform crystals obtained in several tests for acetone by Gunning's method (× about 600).

2. **Lange's Test.**—This is a modification of the well-known Legal test. It is more sensitive and gives a sharper end-reaction. To a small quantity of urine add about one-twentieth its volume (1 drop for each c.c.) of glacial acetic acid and a few drops of fresh concentrated aqueous solution of sodium nitroprussid, and gently run a little ammonia upon its surface. If acetone be present, a reddish-purple ring will form within a few minutes at the junction of the two fluids.

Lange's test is even more sensitive to diacetic acid than to acetone. For this reason **Rothera's test,** which is more sensitive to acetone, is to be preferred: To 5 or 10 c.c. of urine add about a gram of ammonium sulphate and 2 or 3 drops of fresh concentrated sodium nitroprussid solution and overlay with strong ammonia. A reddish-purple ring shows the presence of acetone.

3. **Frommer's Test.**—This test has proved very satisfactory. The

urine need not be distilled. Alkalinize about 10 c.c. of the urine with 2 or 3 c.c. of 40 per cent. caustic soda solution, add 10 or 12 drops of 10 per cent. alcoholic solution of salicylous acid (salicyl aldehyd), heat the upper portion to about 70° C. (it should not reach the boiling-point), and keep at this temperature five minutes or longer. In the presence of acetone an orange color, changing to deep red, appears in the heated portion. A yellow to brown color may appear in the absence of acetone.

The test can be made more definite by adding the caustic soda in substance (about 1 gm.), and before it goes into solution adding the salicyl aldehyd and warming the lower portion of the tube.

(2) **Diacetic (aceto-acetic) acid** occurs in the same conditions as acetone, but has more serious significance. In diabetes its persistent presence is a grave symptom and often forewarns of approaching coma. It rarely or never occurs without acetone.

Detection.—The urine should be fresh. If a preservative must be used, toluene is best.

Gerhardt's Test.—To a few cubic centimeters of the urine add 10 per cent. aqueous solution of ferric chlorid, drop by drop, until the phosphates are precipitated; filter and add more of the ferric chlorid. If diacetic acid be present, the urine will assume a Bordeaux-red color. The test is somewhat more definite if applied by the contact or "ring" method (p. 134).

Similar, although not exactly identical, colors may be produced by other substances, as phenol, salicylates, antipyrin, and sodium bicarbonate. To exclude these fallacies it is necessary, whenever the test gives a red or violet color, to repeat it upon a fresh portion of the urine as follows: To about 5 c.c. of urine in a test-tube add an equal volume of water and boil down to the original volume. Cool and add the ferric chlorid as before. Boiling drives off diacetic acid, hence if the color appears in this second test it was not originally due to diacetic acid. It is not sufficient to boil the urine after the color has been brought out by the ferric chlorid, as is sometimes advised, since the color caused by certain of the drugs will then disappear as well as that caused by diacetic acid. Some prefer to acidify the urine with sulphuric acid, shake out with a little ether, and apply Gerhardt's test to the ether. Diacetic acid and certain drugs give red colors, but that due to diacetic acid fades within twenty-four to forty-eight hours. Whenever the reaction is doubtful, the urine should be distilled and the distillate tested for acetone, or Lindemann's test may be tried.

Lindemann's Test.—To about 10 c.c. of urine add 5 drops of 30 per cent. acetic acid, 5 drops Lugol's solution, and 2 or 3 c.c. chloroform, and shake gently. The chloroform does not change color if diacetic acid be present, but becomes reddish violet in its absence. The test is claimed by its advocates to be more sensitive and more reliable than Gerhardt's. Uric acid also decolorizes iodin, and if much is present double the amount of Lugol's solution should be used.

(3) **Beta-oxybutyric acid** has much the same significance as diacetic acid, but is of more serious import. It is rarely tested for, since it is seldom or never present without acetone and diacetic acid which are more easily detected.

Hart's Test.—Remove acetone and diacetic acid by diluting 20 c.c. urine with 20 c.c. water, adding a few drops of acetic acid, and boiling down to 10 c.c. To this add 10 c.c. water, mix, and divide between two test-tubes. To one tube add 1 c.c. of hydrogen peroxid, warm gently, and cool. This transforms β-oxybutyric acid to acetone. Now apply Lange's test for acetone (p. 154) to each tube. A positive reaction in the tube to which hydrogen peroxid has been added shows the presence of β-oxybutyric acid in the original sample of urine.

4. Bile.—The pigment of bile has its origin in the never-ceasing destruction of red blood-corpuscles within the body.

The significance of bile in the urine is practically the same as that of bile-staining of the tissues, known as icterus or jaundice. Small amounts of bile may, however, be found in the urine when the disturbance is not severe enough to produce recognizable jaundice or, in other cases, before the jaundice supervenes. The usual cause of icterus is some obstruction to the outflow of bile from the liver, which may be in the nature of foreign bodies or new growths inside or outside of the bile passages, or inflammatory swelling of the walls with narrowing of the lumen. Jaundice may also occur when there is excessive destruction of red blood-corpuscles from any cause, notably in the disease known as hemolytic jaundice or congenital familial icterus. This leads to excessive formation of a bile which is more inspissated than normal and thus tends to block the bile-capillaries. The formation of bile in the general circulation in these conditions is also recognized. Another, less frequent, cause of jaundice is rapid destruction of liver cells, as in acute yellow atrophy

and phosphorus-poisoning. Strong emotion has also been known to cause jaundice in some obscure way. Both bile-pigment and bile-acids may be found. They generally occur together, but the pigment is not infrequently present alone.

Of the several pigments, bilirubin alone occurs in freshly voided urine, the others (biliverdin, bilifuscin, and so forth) being produced from this by oxidation as the urine stands. The bile acids, which occur chiefly as sodium salts, are not often present without the pigments, and are, therefore, seldom tested for clinically. Crystals of bilirubin (hematoidin) (Fig. 54, 4) may be deposited in heavily bile-charged urine.

Detection of Bile-pigment.—Bile-pigment gives the urine a greenish-yellow, yellow, or brown color, which upon shaking is imparted to the foam. Cells, casts, and other structures in the sediment may be stained brown or yellow. This, however, should not be accepted as proving the presence of bile without further tests.

1. **Smith's Test.**—Overlay the urine with tincture of iodin diluted with nine times its volume of alcohol. An emerald-green ring at the zone of contact shows the presence of bile-pigments. It is convenient to use a conical test-glass one side of which is painted white.

2. **Gmelin's Test.**—This consists in bringing slightly yellow nitric acid into contact with the urine. A play of colors, of which green and violet are most distinctive, denotes the presence of bile-pigment. Blue and red may be produced by indican and urobilin, violet by iodids. Colorless nitric acid will become yellow upon standing in the sunlight. The test may be applied in various ways: by overlaying the acid with the urine; by bringing a drop of each together upon a porcelain plate; by filtering the urine through thick filter-paper, and touching the paper with a drop of the acid; and, probably best of all, by precipitating with lime-water, filtering, and touching the precipitate with a drop of the acid. In the last method, bilirubin is carried down as an insoluble calcium compound which concentrates the pigment and avoids interfering substances.

Detection of Bile Acids.—Hay's test is simple, sensitive, and fairly reliable, and will, therefore, appeal to the practitioner. It depends upon the fact that bile acids lower surface tension. Other tests require isolation of the acids for any degree of accuracy. Oliver has recently shown that with Heller's test bile acids give a white ring just above that due to albumin.

Hay's Test.—Upon the surface of the urine, *which must not be warm*, sprinkle a little finely powdered sulphur ("flowers of sulphur"). If it sinks at once, bile acids are present to the amount of 0.01 per cent. or more; if only after gentle agitation, 0.0025 per cent. or more. If it remains floating, even after gentle shaking, bile acids are absent. It is said that urobilin when present in large amount also reduces surface tension.

5. Urobilin.—Traces of this pigment, too small for detection by the ordinary tests, are present under normal conditions. It is now regarded as identical with hydrobilirubin, the principal coloring matter of the feces. It is excreted as a chromogen, *urobilinogen*, which is changed into urobilin through the action of light within a few hours after the urine is voided. A great excess gives the urine a dark brown color suggesting the presence of bile, but does not color the foam so deeply as does bile. Small amounts cause no perceptible change in color.

The mode of formation of urobilin is not yet clearly understood. According to the generally accepted view it is a decomposition product of bilirubin, formed chiefly in the intestine through the action of bacteria. Upon the other hand, the formation of small amounts in the liver itself under certain conditions cannot be denied. Urobilinogen is first formed. Under normal conditions a portion of this is absorbed from the intestine, carried to the liver in the portal blood, and there reconverted into bilirubin. When the liver cells are deranged, this transformation into bilirubin does not take place and urobilinogen reaches the general circulation and is excreted by the kidneys. The remainder in the intestine, changed largely into urobilin, passes out with the feces. The pigment and the chromogen have exactly the same significance in the urine, and the name "urobilin" is commonly used to cover both.

Owing to the many unknown factors it is impossible to ascribe definite clinical significance to urobilinuria. Certain statements, however, seem justified. Whenever, owing to excessive destruction of blood-corpuscles, there is excessive formation of bilirubin, and hence an increase of urobilin in the feces, there is also a marked increase in the urine. This is especially important in pernicious anemia and malaria. When not due to excessive blood destruction, urobilinuria usually points toward functional incapacity of the liver. Its recognition is very simple and has considerable practical

usefulness, as, for example, in the diagnosis of hepatic cirrhosis; in judging the amount of damage done to the liver parenchyma by poisons and the chronic congestion of poorly compensated heart disease; and in differentiating anemias associated with excessive blood destruction (for example, pernicious anemia) from those due to other causes (carcinoma, hemorrhage). Urobilinuria is frequent in acute infectious diseases, especially in scarlet fever and pneumonia, and usually means either hemolysis or damage to the liver. In severe nephritis urobilin may fail to be excreted even when other conditions favor its appearance in the urine. It is nearly or entirely absent in obstructive jaundice.

To be of value, tests for urobilin should be made upon several successive days, because for some unknown reason it may be absent for a day or two, and it is advisable to make a simultaneous study of the urobilin of the feces.

1. **Ehrlich's Test for Urobilinogen.**—To a few cubic centimeters of the urine in a test-tube add a few crystals of para-dimethyl-amino-benzaldehyd and make definitely acid with hydrochloric acid. In the presence of pathologic amounts of urobilinogen a cherry-red color appears. This is best seen by viewing the tube from the top over a sheet of white paper. Normal amounts will cause the red color only when the urine is heated.

2. **Schlesinger's Test for Urobilin.**—To about 10 c.c. of the urine in a test-tube add a few drops of Lugol's solution to transform the chromogen into the pigment. Now add about 10 c.c. of a saturated alcoholic solution of zinc acetate or zinc chlorid and filter. A greenish fluorescence, best seen when the tube is viewed in bright sunlight against a black background, and when the light is concentrated upon it with a lens shows the presence of urobilin. The fluorescence becomes more marked after an hour or two. Bile-pigment, if present, should be previously removed by adding about one-fifth volume of 10 per cent. calcium chlorid solution and filtering.

Quantitative Estimation.—The indirect, although clinically satisfactory, method of Wilber and Addis which is given in detail on page 426 may be applied to the urine as follows:

1. To a 10 c.c. portion of the mixed twenty-four-hour urine, which has been preserved with a crystal of thymol and kept in darkness, add 10 c.c. of a saturated alcoholic solution of zinc acetate and filter.

2. To 10 c.c. of the filtrate (representing 5 c.c. of urine) add 1 c.c. of Ehrlich's reagent, the formula for which is as follows:

Para-dimethyl-amino-benzaldehyd	10 gm.
Concentrated hydrochloric acid	75 c.c.
Water	75 "

3. Let stand in the dark for one to three hours, not longer.

4. Examine with a small direct-vision spectroscope and dilute with tap-water until absorption bands disappear. Calculate the total dilution value for the twenty-four-hour quantity of urine as described for urobilin in feces, *basing the calculation upon the 5 c.c. of urine used.*

Example.—If the twenty-four-hour urine amounts to 1200 c.c. and it is necessary to dilute the 10 c.c. filtrate to 50 c.c. to get rid of the absorption bands (supposing that they disappear together), then the combined dilution value for urobilin and urobilinogen in the 5 c.c. of urine would be $10 + 10 = 20$; and the twenty-four-hour value would be $20 \times 240 = 4800$.

6. Hemoglobin.—The presence in the urine of hemoglobin, or pigments directly derived from it, accompanied by few, if any, red corpuscles, constitutes *hemoglobinuria.* It is a comparatively rare condition, and must be distinguished from *hematuria,* or *blood* in the urine, which is common. In both conditions chemic tests will show hemoglobin, but in the latter the microscope will reveal the presence of red corpuscles. Urines which contain notable amounts of hemoglobin have a reddish or brown color, and may deposit a sediment of brown, granular pigment.

Hemoglobinuria occurs when there is such extensive destruction of red blood-cells within the body that the liver cannot transform all the hemoglobin set free into bile-pigment. The most important examples are seen following extensive burns; in poisoning, as by mushrooms and potassium chlorate; in malignant malaria (black-water fever); and in the obscure condition known as "paroxysmal hemoglobinuria." This last is characterized by the appearance of large quantities of hemoglobin at intervals, usually following exposure to cold, the urine remaining free from hemoglobin between the attacks.

Detection.—Teichmann's test may be applied to the precipitate after boiling and filtering, but this is not very satisfactory, and the guaiac or benzidin test will be found more convenient in

routine work. For further discussion of blood tests, including spectroscopic methods, see page 320.

Guaiac Test.—Mix a few cubic centimeters each of "ozonized" turpentine and a fresh 1 : 60 alcoholic solution of gum guaiac. The guaiac solution may be freshly prepared by dissolving a pocket-knife-pointful of powdered guaiac in 4 or 5 c.c. of alcohol. Make the urine strongly acid with acetic acid, and carry out the test by the "ring" or "contact" method described on page 134. A bright blue ring will appear at the zone of contact within a few minutes if hemoglobin be present in considerable amount; a green ring if traces only are present. It may be difficult to get a sharp line of contact, but this is no hindrance since the test is nearly as sharp when the fluids are mixed. The guaiac should be kept in an amber-colored bottle. Fresh turpentine can be "ozonized" by allowing it to stand a few days in an open vessel in the sunlight. Instead of turpentine, hydrogen peroxid may be used.

This test is very sensitive, and a negative result proves the absence of hemoglobin. Positive results are not conclusive, because numerous other substances—few of them likely to be found in the urine—may produce the blue color. That most likely to cause confusion is pus, but the blue color produced by it will appear equally well if the turpentine be omitted, and does not appear if the urine be previously boiled. The thin film of copper often left in a test-tube after testing for sugar may give the reaction, as may also the fumes from an open bottle of bromin. Bromids and iodids likewise give the reaction. Most sources of error can be avoided by extracting the hemoglobin with ether as described on page 321.

Benzidin Test.—The reagents employed are hydrogen peroxid and a saturated solution of benzidin in glacial acetic acid. The benzidin labeled "For blood tests" should be employed. The reagents are mixed in equal parts and a few cubic centimeters are added to a like amount of the urine. A blue color appears in the presence of hemoglobin. The test has the same fallacies as the guaiac test, but is more sensitive and, in general, more satisfactory.

The benzidin test may be simplified by use of the tablets devised by Roberts and put upon the market by the firm of E. R. Squibb & Sons. These contain benzidin and sodium perborate. A tablet is thoroughly moistened with the fluid to be tested and is then touched with a drop of glacial acetic acid, the appearance of a blue color indicating blood. The test is less delicate than those given above.

Spectroscopic Method.—This is discussed on page 322. It is more reliable than the preceding tests, but less sensitive. Render the urine

slightly acid, dilute if very highly colored, and examine with a small direct-vision spectroscope. The usual bands seen are those of oxyhemoglobin or methemoglobin.

To detect traces of blood with the spectroscope proceed as described in Section 2 on page 323. This method will easily detect 2 drops of blood in 8 ounces of urine.

7. Hematoporphyrin is an iron-free pigment derived from hemoglobin. Its formation within the body is not well understood. Normally it appears in the urine only in slight traces. An increase has been observed in a variety of conditions, notably in organic liver diseases, in lead-poisoning, and especially during continued use of sulphonal, trional, and tetronal. In the presence of abnormal amounts the urine may have a dark red or "port wine" color, which, however, appears to be due in part to the simultaneous presence of related but little-known pigments.

It is now recognized that hematoporphyrinuria sometimes occurs as a congenital anomaly of metabolism analogous to cystinuria, alkaptonuria, and congenital pentosuria. In most cases there has been extreme sensitiveness of the skin to sunlight.

Hematoporphyrin does not respond to the guaiac or the hemin test and is best detected with the spectroscope. Treat 100 c.c. of the urine with 20 c.c. of 10 per cent. sodium hydroxid solution. The pigment will be carried down with the phosphates. Filter (or centrifugalize), wash the precipitate with water, then with alchohol, and finally dissolve in about 5 c.c. of alcohol to which 5 to 10 drops of concentrated hydrochloric acid have been added. Examine the acid solution for the absorption bands of acid hematoporphyrin (Fig. 158).

8. Alkapton Bodies.—The name "alkaptonuria" has been given to a condition in which the urine turns reddish brown to brownish black upon standing and strongly reduces copper (but not bismuth), owing to the presence of certain substances which result from imperfect protein metabolism. The chief of these is homogentisic acid. The change of color takes place quickly when fresh urine is alkalinized, hence the name, *alkapton bodies*.

Alkaptonuria is unaccompanied by other symptoms, and has little clinical importance. Only a few cases, mostly congenital, have been reported. The change in color of the urine and the re-

duction of copper, with no reduction of bismuth nor fermentation with yeast, would suggest the condition.

9. Melanin.—Urine which contains melanin likewise darkens upon exposure to the air, assuming a dark brown or black color. This is due to the fact that the substance is eliminated as a chromogen—melanogen—which is later converted into the pigment. It does not reduce copper.

Melanuria occurs in most, but not all, cases of melanotic tumor. Its diagnostic value is lessened by the fact that it has been observed in other diseases, although rarely. It is apparently the result of a type of protein destruction which may occur at times in widely different clinical conditions.

Tests for Melanin.—1. Addition of ferric chlorid gives a gray precipitate which blackens on standing.

2. Bromin-water causes a yellow precipitate which gradually turns black.

10. Diazo Substances.—Certain imperfectly known substances sometimes present in the urine give a characteristic color reaction—the "diazo-reaction" of Ehrlich—when treated with diazo-benzol-sulphonic acid and ammonia. This reaction has considerable clinical value, *provided its limitations be recognized.* It is at best an empirical test and must be interpreted in the light of clinical symptoms. Although it has been met with in a considerable number of diseases, its usefulness is practically limited to typhoid fever, tuberculosis, and measles.

(1) **Typhoid Fever.**—Practically all cases give a positive reaction, which varies in intensity with the severity of the disease. It is so constantly present that it is sometimes said to be "negatively pathognomonic": if negative upon several successive days *at a stage of the disease when it should be positive,* typhoid is almost certainly absent. Upon the other hand, a reaction when the urine is highly diluted (1 : 50 or more) has much positive diagnostic value, since this dilution prevents the reaction in most conditions which might be mistaken for typhoid; but it should be noted that mild cases of typhoid may not give it at this dilution. Ordinarily the reaction appears about the fourth or fifth day of the disease. It begins to fade about the end of the second week, and soon thereafter entirely disappears. An early disappearance is a favorable

sign. It reappears during a relapse, and thus helps to distinguish between a relapse and a complication, in which it does not reappear.

(2) **Tuberculosis.**—The diazo-reaction has been obtained in many forms of the disease. It has little or no diagnostic value. Its continued presence in pulmonary tuberculosis is, however, a grave prognostic sign, even when the physical signs are slight. After it once appears it generally persists more or less intermittently until death, the average length of life after its appearance being about six months. The reaction is often temporarily present in mild cases during febrile complications, and has then no significance.

(3) **Measles.**—A positive reaction is usually obtained in measles, and may help to distinguish this disease from German measles, in which it does not occur. It generally appears before the eruption and remains about five days.

Technic.—Although the test is really a very simple one, careful attention to technic is imperative. Many of the early workers were very lax in this regard. Faulty technic and failure to record the stage of the disease in which the tests were made have probably been responsible for the bulk of the conflicting results reported. Reactions more or less closely simulating the diazo-reaction have been observed after administration of naphthalin, opium, and its alkaloids, atophan, salol, creosote, phenol, and the iodids. Tannic acid and its compounds prevent the reaction.

The reagents are:

(1) Sulphanilic acid	1.0 gm.
Concentrated hydrochloric acid	10.0 c.c.
Water	200.0 "
(2) Sodium nitrite	0.5 gm.
Water	100.0 c.c.
(3) Strong ammonia.	

Mix 100 parts of (1) and 1 part of (2).[1] In a test-tube take equal parts of this mixture and the urine, and pour 1 or 2 c.c. of the ammonia upon its surface. If the reaction be positive, a garnet ring will form at the junction of the two fluids, and, upon shaking, a distinct pink color will be imparted to the foam. The color of the foam is the essential

[1] These proportions are recommended by Greene, and are now generally used. Ehrlich used 40 parts of (1) and 1 part of (2).

feature. If desired, the mixture may be well shaken before the ammonia is added: the pink color will then instantly appear in that portion of the foam which the ammonia has reached, and can be readily seen. The color varies from eosin-pink to deep crimson, depending upon the intensity of the reaction. *It is a pure pink or red; any trace of yellow or orange denotes a negative reaction.* A doubtful reaction should be considered negative.

Weis' Urochromogen Test.—Weis believed the diazo-reaction to be due principally to urochromogen, which, because of the effect of certain toxins upon metabolism, fails of conversion into urochrome; and he offered this permanganate reaction as a more satisfactory test, both for urochromogen and for an antecedent substance which has the same significance as urochromogen, but which the diazo fails to detect. This test has been studied chiefly in its relation to prognosis in tuberculosis, in which it appears to have about the same value as the diazo, with the differences that it is more frequently noted and is less intermittent in a given case and probably has less serious import.

In a test-tube mix 2 c.c. of urine and 4 c.c. distilled water, and add 3 drops of 1 : 1000 aqueous solution of potassium permanganate. The appearance of a yellow color denotes a positive reaction. The color is best judged by comparison with a tube of diluted urine to which no permanganate has been added, the two tubes being viewed from the top over a sheet of white paper. The color of a genuine reaction is a canary yellow. A yellow color, usually not so bright, and tending more toward brown, may be produced by urobilin and other substances, but these false reactions fade quickly, usually within thirty seconds, while the color of a true reaction remains a longer time.

11. Drugs.—The effect of various drugs upon the color of the urine has been mentioned (p. 87). Most poisons are eliminated in the urine, but their detection is more properly discussed in works upon toxicology. A few drugs which are of interest to the practitioner, and which can be detected by comparatively simple methods, are mentioned here.

Acetanilid and Phenacetin.—The urine is evaporated by gentle heat to about half its volume, boiled for a few minutes with about one-fifth its volume of strong hydrochloric acid, and shaken out with ether. The ether is evaporated, the residue dissolved in water, and the following test applied: To about 10 c.c. are added a few cubic centimeters of 3 per cent. phenol followed by a weak solution of chromium trioxid (chromic acid) drop by drop. The

fluid assumes a red color, which changes to blue when ammonia is added. If the urine be very pale, extraction with ether may be omitted.

Antipyrin.—This drug gives a dark red color when a few drops of 10 per cent. ferric chlorid are added to the urine. The color does not disappear upon boiling, which excludes diacetic acid.

Arsenic.—*Reinsch's Test.*—Add to the urine in a test-tube or small flask about one-seventh its volume of hydrochloric acid, introduce a piece of bright copper-foil about 3 mm. square, and boil for several minutes. If arsenic be present, a dark gray film is deposited upon the copper. The test is more delicate if the urine be concentrated by slow evaporation. This test is well known and is widely used, but is not so reliable as the following:

Gutzeit's Test.—In a large test-tube place a little arsenic-free zinc, and add 5 to 10 c.c. pure dilute hydrochloric acid and a few drops of iodin solution (Gram's solution will answer), then add 5 to 10 c.c. of the urine. At once cover the mouth of the tube with a filter-paper cap moistened with saturated aqueous solution of silver nitrate (1 : 1). If arsenic be present, the paper quickly becomes lemon yellow, owing to formation of a compound of silver arsenid and silver nitrate, and turns black when touched with a drop of water. To make sure that the reagents are arsenic free, the paper cap may be applied for a few minutes before the urine is added.

Atropin will cause dilatation of the pupil when a few drops of the urine are placed in the eye of a cat or rabbit.

Bromids can be detected by acidifying about 10 c.c. of the urine with dilute sulphuric acid, adding a few drops of fuming nitric acid and a few cubic centimeters of chloroform, and shaking. In the presence of bromin the chloroform, which settles to the bottom, assumes a yellow color.

Chloral hydrate appears in the urine chiefly as urochloralic acid, which reduces the copper solutions used for sugar tests. To detect it, evaporate about 500 c.c. of the urine to about one-fourth its volume, make decidedly acid with hydrochloric acid, add about 50 c.c. of ether, shake thoroughly, and separate the ether. Now evaporate the ether and dissolve the residue in a little water. If urochloralic acid be present, this aqueous solution will respond to Fehling's test.

Formaldehyd is discussed under Hexamethylenamin.

Hexamethylenamin.—Interest in this drug centers chiefly in its value as a urinary antiseptic, which depends upon its decomposition in acid urine with liberation of formaldehyd. According to a number of recent workers formaldehyd can be detected in the urine of only about 50 per cent. of patients who are taking hexamethylenamin. A test for formaldehyd is, therefore, necessary in order to know whether the object in administering the drug is being accomplished.

Jorissen's Test for Formaldehyd.—To 1 or 2 c.c. of the urine or other fluid to be tested add about 0.5 c.c. of a 1 per cent. solution of phloroglucinol (Merck's "reagent") in 10 per cent. sodium hydroxid. The appearance of a bright red color shows the presence of free formaldehyd. The test is said to detect 1 part in 10,000,000.

Iodin, from ingestion of iodids or absorption from iodoform dressings, is tested for in the same way as the bromids, the chloroform assuming a pink to reddish-violet color; or Obermayer's reagent may be used in the same way as described for indican (p. 119). To detect traces a large quantity of urine should be rendered alkaline with sodium carbonate and greatly concentrated by evaporation before testing.

Lead.—No simple method is sufficiently sensitive to detect the traces of lead which occur in the urine in chronic poisoning. Of the more sensitive methods, that of Arthur Lederer is perhaps best suited to the practitioner:

It is essential that all apparatus used be lead free: 500 c.c. of the urine are acidified with 70 c.c. of pure sulphuric acid and heated in a beaker or porcelain dish. About 20 to 25 gm. of potassium persulphate are added a little at a time. This should decolorize the urine, leaving it only slightly yellow. If it darkens upon heating, a few more crystals of potassium persulphate are added, the burner being first removed to prevent boiling over; if it becomes cloudy, a small amount of sulphuric acid is added. It is then boiled until it has evaporated to 250 c.c. or less. After cooling, an equal volume of alcohol is added, and the mixture allowed to stand in a cool place for four or five hours, during which time all the lead will be precipitated as insoluble sulphate.

The mixture is then filtered through a small, close-grained filter-paper (preferably an ashless, quantitative filter-paper), and any sediment remaining in the beaker or dish is carefully washed out with alcohol and filtered. A test-tube is placed underneath the funnel; a hole is punched through the tip of the filter with a small glass rod, and all the precipitate (which may be so slight as to be scarcely visible) washed down into the test-tube with a jet of distilled water from a wash-bottle, using as little water as possible; 10 c.c. will usually suffice. This fluid is then heated, adding crystals of sodium acetate until it becomes perfectly clear. It now contains all the lead of the 500 c.c. urine in the form of lead acetate. It is allowed to cool, and hydrogen sulphid gas is passed through it for about five minutes. The slightest yellowish-brown discoloration indicates the presence of lead. A very slight discoloration can be best seen when looked at from above. For comparison, the gas may be passed through a test-tube containing an equal amount of distilled water. The quantity of lead can be determined by comparing the discoloration with that produced by passing the gas through lead acetate (sugar of lead) solutions of known strength. One gram of lead acetate crystals contains 0.54 gm. of lead. Hydrogen sulphid is easily prepared in the simple apparatus shown in Figure 53. A small quantity of iron sulphid is placed in the test-tube, a little dilute hydrochloric acid is added, the cork is replaced, and the delivery tube is inserted to the bottom of the fluid to be tested.

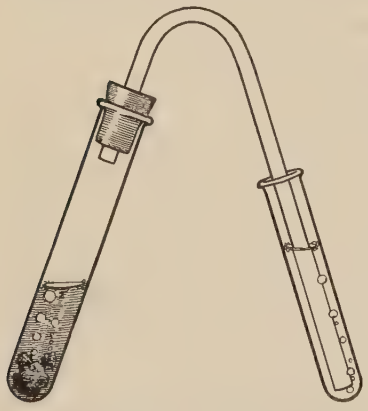


FIG. 53.—A simple hydrogen sulphid generator described in the text.

Mercury (*Method of Vogel and Lee*).—This will detect 1 mg. of mercury in 100 c.c. of urine, gastric contents, or feces.

To about 150 c.c. of the urine add about 5 c.c. of concentrated hydrochloric acid and boil down to 25 or 30 c.c. Add about 2 c.c. of hydrochloric acid to replace that lost in boiling and enough potassium chlorate, usually about 2 gm., to oxidize the organic matter, as shown by a change of color to pale yellow. Dilute to about 60 c.c. and boil a few minutes to drive off chlorin.

Secure a piece of copper wire of about 18 gage and 4 cm. long, bend it several times upon itself, clean by boiling a few minutes in dilute hydrochloric acid, and with the aid of forceps place it in the concentrated urine, where it should remain for two hours. In the presence of mercury it will become coated with a silvery film. Next remove the wire with the aid of forceps, rinse in water, dry, and place it in the bottom of a slender test-tube, made by sealing one end of a piece of glass tubing about 15 cm. long and 3 to 5 mm. in diameter. Also insert in the tube a plug of gold leaf and push it down to about 2 mm. from the wire. The gold leaf is that used in dentistry. Holding the tube horizontally, gently heat the end containing the wire, but avoid heating the plug of gold leaf. Examine the gold leaf frequently during the heating, preferably with a hand lens. If mercury be present, it will form a silvery patch of amalgum on the end of the gold plug toward the wire. Vogel and Lee recommend that in important cases the tube and its contents be kept as a permanent record after sealing the open end.

For confirmation the gold plug may be removed and suspended in a test-tube containing a crystal of iodin which is gently heated. The silvery deposit of mercury is changed to red iodid of mercury.

Morphin.—Add sufficient ammonia to the urine to render it distinctly ammoniacal, and shake thoroughly with a considerable quantity of pure acetic ether. Separate the ether and evaporate to dryness. To a little of the residue in a watch-glass or porcelain dish add a few drops of formaldehyd-sulphuric acid, which has been freshly prepared by adding 1 drop of formalin to 1 c.c. pure concentrated sulphuric acid. If morphin be present, this will produce a purplish-red color, which changes to violet, blue violet, and finally nearly pure blue.

Phenol.—As has been stated, the urine following phenol-poisoning turns olive green and then brownish black upon standing. Tests are of value in recognizing poisoning from ingestion and in detecting absorption from carbolized dressings.

The urine is strongly acidified with hydrochloric acid and distilled. To the first few cubic centimeters of distillate is added 10 per cent. solution of ferric chlorid, drop by drop. The presence of phenol causes a deep amethyst-blue color, as in Uffelmann's test for lactic acid (p. 399).

Phenolphthalein, which is now widely used as a cathartic, gives a bright pink color when the urine is rendered alkaline.

Quinin.—A considerable quantity of the urine is rendered alkaline with ammonia and extracted with ether; the ether is evaporated, and a portion of the residue dissolved in about 20 drops of dilute alcohol. The alcoholic solution is acidulated with dilute sulphuric acid, 1 drop of án alcoholic solution of iodin (tincture of iodin diluted about ten times) is added, and the mixture is warmed. Upon cooling, an iodin compound of quinin (herapathite) will separate out in the form of a microcrystalline sediment of green plates.

The remainder of the residue may be dissolved in a little dilute sulphuric acid. This solution will show a characteristic blue fluorescence when quinin is present.

Resinous drugs cause a white precipitate like that of albumin when strong nitric acid is added to the urine. This is dissolved by alcohol.

Salicylates, salol, aspirin, and similar drugs give a bluish-violet color upon addition of a few drops of 10 per cent. ferric chlorid solution to the urine, which must previously be boiled to drive off any diacetic acid that may be present. When the quantity of salicylates is small, the urine may be acidified with hydrochloric acid and extracted with ether, the ether evaporated, and the test applied to an aqueous solution of the residue.

Tannin and its compounds appear in the urine as gallic acid, and the urine becomes greenish black (inky if much gallic acid be present) when treated with a solution of ferric chlorid.

IV. MICROSCOPIC EXAMINATION

A careful microscopic study should be a part of every routine urine examination. It will often reveal structures of diagnostic importance in urine which seems perfectly clear, and from which only very slight sediment can be obtained with the centrifuge. Upon the other hand, cloudy urines with abundant sediment are often shown by the microscope to contain no structures of clinical significance.

Since the nature of the sediment soon changes, the urine must be examined while fresh, preferably within six hours after it is voided. If it must be kept for a much longer period some pre-

servative should be added, preferably 4 drops of formalin, or 5 grains of boric acid, or 1 c.c. of toluene for each 4 ounces of urine. When possible it should be placed on ice. The sediment is best obtained by means of the centrifuge. If a centrifuge is not available, the urine may be allowed to stand in a conical test-glass for six to twenty-four hours.

A small amount of the sediment should be transferred to a slide by means of a pipet. It is very important to do this properly. The best pipet is a simple glass tube, 7 or 8 inches long, which has been drawn out at one end to a tip with a 1 or 1.5 mm. opening. The centrifuge tube containing the sediment is held on a level with the eye, the larger end of the pipet is closed with the index-finger, which must be dry, and the tip is carried down into the sediment. By carefully loosening the finger, but not entirely removing it, a small amount of the sediment is then allowed to run slowly into the pipet. Slightly rotating the pipet will aid in accomplishing this, and at the same time will serve to loosen any structures which cling to the bottom of the tube. After wiping off the urine which adheres to the outside, a drop from the pipet is placed upon a clean slide. A hair is then placed in the drop and a large cover-glass applied. The correct size of the drop can be learned only by experience. It should not be so large as to float the cover-glass about, or so small as to leave unoccupied space beneath the cover. Many workers use no cover. This offers a thicker layer and larger area of urine, the chance of finding scanty sctructures being proportionately increased. It has the disadvantage that any jarring of the room (as by persons walking about) sets the microscopic field into vibratory motion and makes it impossible to see anything clearly; and, since it does not allow satisfactory use of high-power objectives, one cannot examine details as carefully as one often wishes to do. It is true that a cover can be applied later, but any structure which one has found with the low power and wishes to study with the high is sure to be lost when the cover is applied. A cover-glass (about 22 mm. square) with a hair beneath it avoids these disadvantages, and gives enough urine to find any structures which are present in sufficient number to have clinical significance, provided other points in the technic have been right. It is best, however, to examine several drops, and, when the sediment is abundant, drops from the upper and lower portions should be examined

separately. The hair is dispensed with in routine work, but its use should be required in class exercises, since it is a useful aid in teaching the correct thickness of the layer of urine, and the hair serves as a conspicuous object upon which to focus when few structures are to be found.

In examining urinary sediments microscopically no fault is so common or so fatal to good results as improper illumination (Fig. 6), and none is so easily corrected. The light should be central and very subdued for ordinary work, but oblique illumination, obtained by swinging the mirror a little out of the optical axis, will be found helpful in identifying certain delicate structures like hyaline casts. The 16-mm. objective should be used as a finder, while the 4-mm. is reserved for examining details. An experienced worker will rely almost wholly upon the lower power.

It is well to emphasize that *the most common errors which result in failure to find important structures, when present, are: (a) lack of care in transferring the sediment to the slide; (b) too strong illumination, and (c) too great magnification.*

In order to distinguish between similar structures it is often necessary to watch the effect upon them of certain reagents. This is especially true of the various unorganized sediments. They very frequently cannot be identified from their form alone. With the structures still in focus, a drop of the reagent may be placed at one edge of the cover-glass and drawn underneath it by the suction of a piece of blotting-paper touched to the opposite edge; or, better, a small drop of the reagent and of the urine may be placed close together upon a slide and a cover gently lowered over them. As the two fluids mingle, the effect upon various structures may be seen.

A common error is the attempt to identify objects in urine which has dried upon the slide. Satisfactory examination is impossible under such conditions. Not only are the delicate organized structures distorted beyond recognition, but there is a confusing deposit of urinary salts. After a little experience one recognizes at a glance from the peculiar refraction of the structures seen that the urine has dried.

The record of the microscopic examination should not merely state that particular structures are present, but should give an approximate idea of their number. The best plan is to record the

average number seen in a field of the low-power objective, although the number will vary greatly with the thoroughness of centrifugation, and especially with the care with which the sediment is transferred to the slide. The approximate amount of sediment in the centrifuge tube should also be recorded.

Urinary sediments may be studied under three heads: A. Unorganized sediments. B. Organized sediments. C. Extraneous structures.

A. Unorganized Sediments

In general, these have little diagnostic or prognostic significance. Most of them are substances normally present in solution, which have been precipitated either because present in excessive amounts, or, more frequently, because of some alteration in the urine

Fig. 54.—Unusual urinary crystals (drawn from various authors): 1, Calcium sulphate (colorless); 2, cholesterol (colorless); 3, hippuric acid (colorless); 4, hematoidin (brown); 5, fatty acids (colorless); 6, indigo (blue); 7, sodium urate (yellowish).

(as in reaction, concentration, and so forth) which may be purely physiologic, depending upon changes in diet or habits. Various substances are always precipitated during decomposition, which may take place either within or without the body.

Unorganized sediments may be classified according to the reaction of the urine in which they are *most likely* to be found. This classification is useful, but is not accurate, since the characteristic sediments of acid urine may remain after the urine has become alkaline, while the alkaline sediments may be precipitated in a urine which is still acid.

In Acid Urine.—Uric acid, amorphous urates, sodium urate, calcium oxalate, leucin and tyrosin, cystin, and fat-globules may be found. Uric acid, the urates, and calcium oxalate are the common

deposits of acid urines; the others are less frequent, and depend less upon the reaction of the urine.

In Alkaline Urine.—Phosphates, calcium carbonate, and ammonium biurate may be deposited.

Other crystalline sediments (Fig. 54) which are rare and require no further mention are: Calcium sulphate, cholesterol, hippuric acid, hematoidin, fatty acids, and indigo.

The following brief table will aid the student in identifying the chemical sediments which one meets every day:

	In acid urine.	In alkaline urine.
Yellow crystals.....	Uric acid—dissolve in NaOH.	Ammonium biurate—dissolve in HCl.
Colorless crystals.....	Calcium oxalate—dissolve in HCl.	Phosphate crystals—dissolve in acetic acid.
Amorphous material.....	Urates—dissolve with heat.	Amorphous phosphates—dissolve in acetic acid.

1. In Acid Urine.—(1) **Uric-acid Crystals.**—These crystals are the red grains—"gravel" or "red sand"—which are often seen adhering to the sides and bottom of a vessel containing urine. Microscopically, they are yellow or reddish-brown crystals, which differ greatly in size and shape. The color is due to urinary pigments, chiefly uro-erythrin. The most characteristic forms (Plate III and Fig. 55) are "whetstones"; roset-like clusters of prisms and whetstones; and rhombic plates, which have usually a paler color than the other forms and are sometimes colorless. A very rare form is a colorless hexagonal plate resembling cystin. Recognition of the crystals depends less upon their shape than upon their color, the reaction of the urine, and the facts that they are soluble in sodium hydroxid solution and insoluble in hydrochloric or acetic acid. When ammonia is added, they dissolve and crystals of ammonium urate appear.

A deposit of uric acid crystals has no significance unless it occurs before or very soon after the urine is voided. Every urine, if kept acid, will in time deposit its uric acid. Factors which favor an early deposit are high acidity, diminished urinary pigments, and

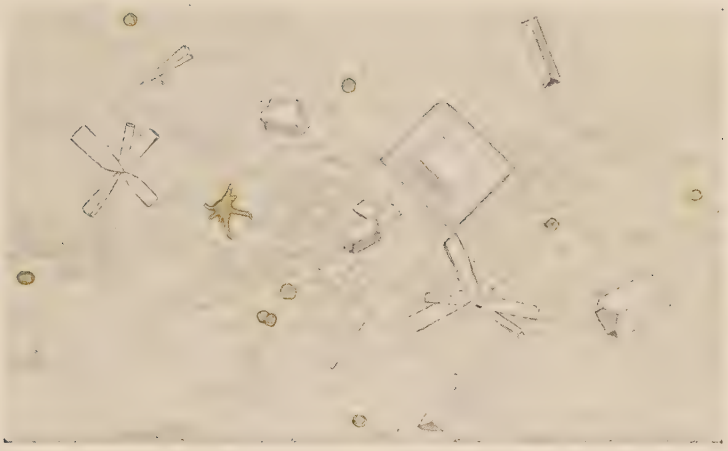


FIG. 1.—Common sediments of alkaline urine: Triple phosphate crystals, calcium phosphate crystals, ammonium urate crystals, and amorphous phosphates. (× 150.)

FIG. 2.—Common sediments of acid urine: Uric-acid crystals, calcium oxalate crystals, and amorphous urates. (× 150.)

excessive excretion of uric acid. The chief clinical interest of the crystals lies in their tendency to form calculi, owing to the readiness with which they collect about any solid object. Their presence in the freshly voided urine in clusters of crystals suggests stone in the kidney or bladder, especially if blood is also present (Fig. 91).

It was formerly believed that the uric acid stone is the most common form of renal calculus, but from a recent study of a series of calculi Kahn and Rosenbloom believe that the great majority are composed of calcium oxalate, although all contain a trace of uric acid.

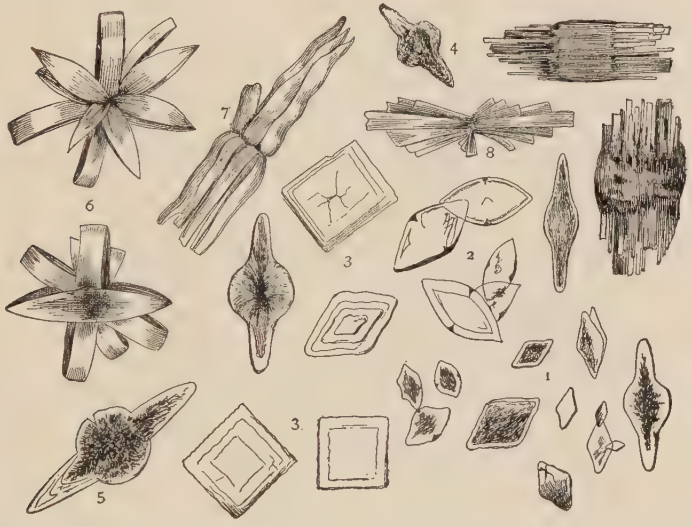


Fig. 55.—Forms of uric acid: 1. Rhombic plates; 2, "whetstone" forms; 3, 3, quadrate forms; 4, 5, prolonged into points; 6, 8, rosets; 7, pointed bundles; 8, barrel forms precipitated by adding hydrochloric acid to urine. (Ogden.)

(2) **Amorphous Urates.**—These are chiefly urates of sodium and potassium which are thrown out of solution as a yellow or red "brick-dust" deposit. In pale urines this sediment is almost white. It disappears upon heating. A deposit of amorphous urates is very common in concentrated and strongly acid urines, especially in cold weather, and has no clinical significance. It is particularly frequent in febrile conditions. Under the microscope it appears as fine yellowish granules, sometimes almost colorless (Plate III). Often they are so abundant as to obscure all other structures. In such cases the urine should be warmed before examining. The

granules have a tendency to collect upon tube-casts, strands of mucus, and other structures. Amorphous urates are readily soluble in caustic soda solutions. When treated with hydrochloric or acetic acid, they slowly dissolve and rhombic crystals of uric acid appear in ten to twenty minutes.

Rarely sodium urate occurs in crystalline form—slender prisms, arranged in fan- or sheaf-like structures (Fig. 54).

(3) **Calcium Oxalate.**—Characteristic of calcium oxalate are colorless, glistening, octahedral crystals, giving the appearance of small squares crossed by two intersecting diagonal lines—the so-called "envelope crystals" (Fig. 86). They vary greatly in size, being sometimes so small as to seem mere points of light with

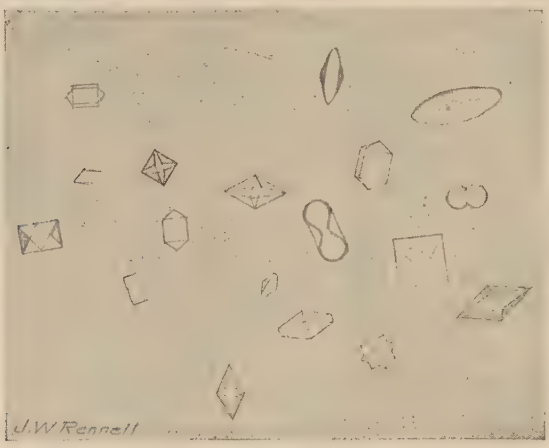


FIG. 56.—Various forms of calcium oxalate crystals from urine. The majority are the typical octahedra seen in different positions (× 450).

medium-power objectives. Unusual forms are colorless dumb-bells, spheres, and variations of the octahedra (Fig. 56). The spheres might be mistaken for globules of fat or red blood-corpuscles. Crystals of calcium oxalate are insoluble in acetic acid or caustic soda. They are dissolved by strong hydrochloric acid, and re-crystallize as octahedra upon addition of ammonia. They are sometimes encountered in alkaline urine.

The crystals are commonly found in the urine after ingestion of vegetables rich in oxalic acid, as tomatoes, spinach, asparagus, and rhubarb. They have no definite significance pathologically. They often appear in digestive disturbances with fermentation of carbohydrates, in neurasthenia, and when the oxidizing power of the

system is diminished. When abundant they are generally associated with a little mucus; and, in men, frequently with a few spermatozoa. Their chief clinical interest lies in their tendency to form calculi, and their presence in fresh urine, together with evidences of renal or cystic irritation, should be viewed with suspicion, particularly if they are clumped in small masses.

(4) **Leucin and Tyrosin.**—These substances are cleavage products of the protein molecule. They are of comparatively rare occurrence in the urine and generally appear together. In general, their presence indicates autolysis of tissue proteins. Clinically, they are seen most frequently in severe fatty destruction of the liver, such as occurs in acute yellow atrophy and phosphorus-poisoning. Crystals are deposited spontaneously only when the substances are present in large amount. Usually they will be deposited when the urine is evaporated to a small volume on a water-bath. It is best, however, to separate them from the urine as follows:

Treat 500 to 1000 c.c. of urine, which has been freed from albumin, with neutral, then with basic, lead acetate until a precipitate no longer forms. Filter, precipitate excess of lead with hydrogen sulphid (p. 168), and filter again. Concentrate to a syrup on a water-bath. Extract repeatedly with small quantities of absolute alcohol to remove urea. Treat the residue with hot dilute alcohol to which a little ammonia has been added. Filter and evaporate the filtrate to a small volume and let stand for the leucin and tyrosin to separate out. The leucin can be separated from the tyrosin by boiling with glacial acetic acid. Leucin dissolves, leaving the tyrosin, and can again be recovered by evaporating the acetic acid.

The crystals cannot be identified from their morphology alone, since other substances, notably calcium phosphate (Fig. 62) and ammonium biurate, may take similar or identical forms. It is, therefore, necessary to try out their solubility in various reagents or to apply special tests.

Leucin crystals (Fig. 57) as they appear in the urine do not represent the pure substance. They are slightly yellow, oily looking spheres, many of them with radial and concentric striations. Some may be merged together in clusters. They are not soluble in hydrochloric acid or in ether.

Tyrosin crystallizes in very fine needles, which may appear black and which are usually arranged in sheaves, with a marked constriction at the middle (Fig. 58). It is soluble in ammonia and hydrochloric acid, but not in acetic acid.

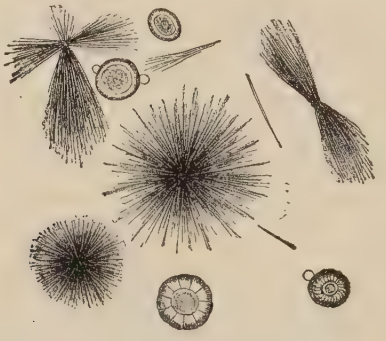


FIG. 57.—Leucin spheres and tyrosin needles (Stengel).

Mörner's Test for Tyrosin.—To a small quantity of the crystals in a test-tube add a few cubic centimeters of Mörner's reagent (formalin, 1 c.c.; distilled water, 45 c.c.; concentrated sulphuric acid, 55 c.c.). Heat gently to the boiling-point. A green color shows the presence of tyrosin.

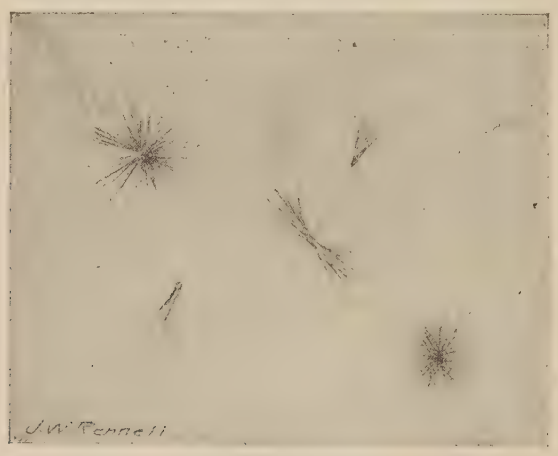


FIG. 58.—Tyrosin crystals from urine (× 450).

(5) **Cystin crystals** are colorless, highly refractive, rather thick, hexagonal plates with well-defined edges. They lie either singly or superimposed to form more or less irregular clusters (Fig. 59). Uric acid sometimes takes this form and must be excluded. Cystin

is soluble in hydrochloric acid, insoluble in acetic; it is readily soluble in ammonia and recrystallizes upon addition of acetic acid.

Cystin is one of the amino-acids formed in decomposition of the protein molecule, and is present in traces in normal urine. Crystals are deposited only when the substance is present in excessive amount. Their presence is known as *cystinuria*. It is a rare condition, due to an obscure abnormality of protein metabolism and usually continues throughout life. The amount of cystin can be greatly diminished by a low-protein diet, and the formation of crystals can in some measure be prevented by administration of sodium carbonate (Smillie). There are rarely any symptoms save

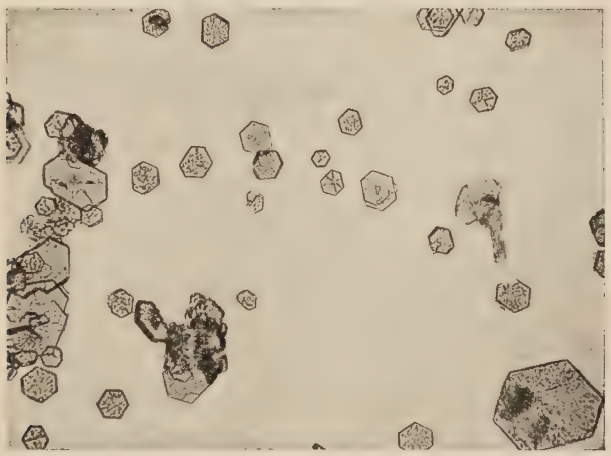


FIG. 59.—Cystin crystals from urine of patient with cystin calculus. The crystals are colorless, with a luster suggesting mother of pearl (photograph; × 200).

those referable to renal or cystic calculus, to which the condition strongly predisposes.

(6) **Fat-globules.**—Fat appears in the urine as highly refractive globules of various sizes, frequently very small. These globules are easily recognized from the fact that they are stained black by osmic acid and orange or red by sudan III or scharlach R. The stain may be applied upon the slide, as already described (p. 172). Osmic acid should be used in 1 per cent. aqueous solution; formulæ for sudan III and scharlach R are given on page 699.

Fat in the urine is usually a contamination from unclean vessels, oiled catheters, or similar sources. A very small amount may be present after ingestion of large quantities of cod-liver oil or other fats.

In fatty degeneration of the kidney, as in phosphorus-poisoning and chronic parenchymatous nephritis, fat-globules are commonly seen, both free in the urine and embedded in cells and tube-casts. Fat-droplets are common in pus-corpuscles and in degenerating cells of any kind.

In *chyluria*, or admixture of chyle with the urine as a result of rupture of a lymph-vessel, minute droplets of fat are so numerous as to give the urine a milky appearance. The droplets are smaller than those of milk, which is sometimes added by malingerers. The fluid is often blood-tinged. The condition is best recognized by shaking with ether, which, when separated, leaves the urine comparatively clear. If, then, the ether be evaporated, a fatty

FIG. 60.—Prismatic forms of triple phosphate crystals from urine (× 450).

residue remains. Chyluria occurs most frequently as a symptom of infection by filaria (p. 502), the larvæ of which can usually be found in the milky urine. In other cases the etiology is obscure.

2. In Alkaline Urine.—(1) **Phosphates.**—While most common in alkaline urine, phosphates are sometimes deposited in amphoteric or feebly acid urines. The usual forms are: (*a*) ammoniomagnesium phosphate crystals; (*b*) dicalcium phosphate crystals, and (*c*) amorphous phosphates. All are readily soluble in acetic acid.

(*a*) *Ammoniomagnesium Phosphate Crystals.*—They are the common "triple phosphate" crystals, which are generally easily recognized (Figs. 60, 61, 92, and Plate III). They are colorless except

when bile stained. Their usual form is some modification of the prism, with oblique ends. Most typical are the well-known "coffin-lid" and "hip-roof" forms. The long axis of the hip-roof crystal is often so shortened that it resembles the envelope crystal of calcium

FIG. 61.—Triple phosphate crystals: forms produced by rapid precipitation and by partial solution of prisms (× 450).

oxalate. It does not, however, have the same luster; this, and its solubility in acetic acid, will always prevent confusion.

When rapidly deposited, as by artificial precipitation, triple phosphate often takes feathery, star-, or leaf-like forms (Fig. 61).

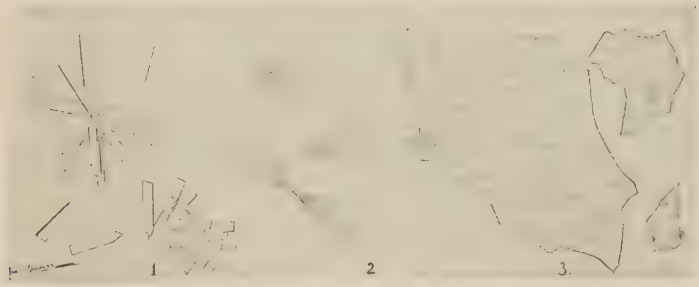


FIG. 62.—Crystals of calcium phosphate: 1, Common form (copied from Rieder's Atlas); 2, needles resembling tyrosin (drawn from nature); 3, large, irregular plates (from nature).

These gradually develop into the more common prisms. X-forms may be produced by partial solution of prisms.

(*b*) *Dicalcium Phosphate Crystals.*—In feebly acid, amphoteric, or feebly alkaline urines dicalcium phosphate is not infrequently desposited in the form of colorless prisms arranged in stars and

rosets (Fig. 62, 1). Because of the shape of the crystals it is sometimes called "stellar phosphate." The individual prisms are usually slender, with one beveled, wedge-like end, but are sometimes needle-like. They may sometimes take forms resembling tyrosin (Fig. 62, 2), calcium sulphate, or hippuric acid, but are readily distinguished by their solubility in acetic acid.

FIG. 63.—Indistinct crystalline sediment (dumb-bell crystals) of calcium carbonate. Similar crystals are sometimes formed by calcium oxalate and calcium sulphate (after Funke).

Calcium phosphate often forms large, thin, irregular, usually granular, colorless plates (Fig. 62, 3) which should be easily recognized, although small plates might be mistaken for squamous epithelial cells. These crystals most frequently form a scum upon the surface of the urine. They are regarded by some as magnesium phosphate.

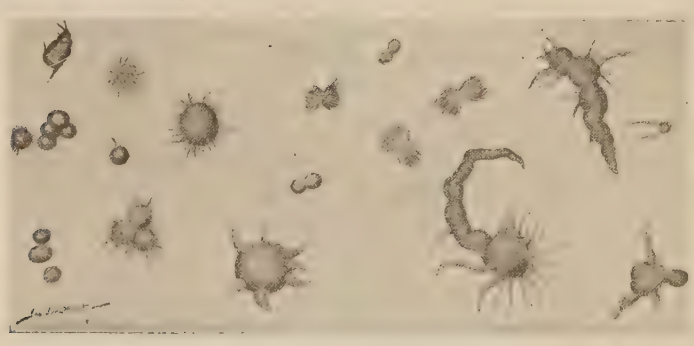


FIG. 64.—Crystals of ammonium biurate (one-half of the forms copied from Rieder's Atlas; the others, from nature).

(*c*) *Amorphous Phosphates.*—The earthy phosphates are thrown out of solution in most alkaline and many amphoteric urines as a white, amorphous sediment, which may be mistaken for pus macroscopically. Under the microscope the sediment is seen to consist of numerous colorless granules, distinguished from amorphous urates by their color, their solubility in acetic acid, and the reaction of the urine.

The various phosphatic deposits frequently occur together. They are sometimes due to excessive excretion of phosphoric acid, but usually merely indicate that the urine has become, or is becoming, alkaline (Phosphates, p. 116).

(2) **Calcium carbonate** may sometimes be mingled with the phosphatic deposits, usually as amorphous granules, or, more rarely, as colorless spheres and dumb-bells (Fig. 63), which are soluble in acetic acid with gas formation.

(3) **Ammonium Biurate Crystals.**—This is the only urate deposited in alkaline urine. It forms opaque yellow crystals, usually in the form of spheres (Plate III and Fig. 92), which are often covered with fine or coarse spicules—"thorn-apple crystals." Sometimes dumb-bells, compact sheaves of fine needles, and irregular rhizome forms are seen (Fig. 64). Upon addition of acetic acid they dissolve, and rhombic plates of uric acid appear.

These crystals occur only when free ammonia is present. They are generally found along with the phosphates in decomposing urine and have no clinical significance.

B. Organized Sediments

The principal organized structures in urinary sediments are: Tube-casts; epithelial cells; pus-corpuscles; red blood-corpuscles; spermatozoa; bacteria, and animal parasites. They are much more important than the unorganized sediments just considered.

1. Tube-casts.—These interesting structures are albuminous casts of the uriniferous tubules. Their presence in the urine (known as *cylindruria*) probably always indicates some pathologic change in the kidney, although this change may be very slight or transitory. Large numbers may be present in temporary irritations and congestions. *They do not in themselves, therefore, imply organic disease of the kidney.* They rarely occur in urine which does not contain, or has not recently contained, albumin, and in a general way have the same clinical significance as renal albuminuria.

While it is not possible to draw a sharp dividing line between the different varieties, casts may be classified as follows:

(1) Hyaline casts:
  (*a*) Narrow.
  (*b*) Broad.
(2) Waxy casts.

(3) Fibrinous casts.
(4) Granular casts:
  (*a*) Finely granular.
  (*b*) Coarsely granular.
(5) Fatty casts.
(6) Casts containing organized structures:
  (*a*) Epithelial casts.
  (*b*) Blood-casts.
  (*c*) Pus-casts.
  (*d*) Bacterial casts.

As will be seen later, practically all varieties are modifications of the hyaline. Not infrequently two varieties are included in the same cast.

The significance of the different varieties is more readily understood if one considers their mode of formation. Albuminous material, the source and nature of which are not definitely known but which are doubtless not the same in all cases, probably enters the lumen of a uriniferous tubule in a fluid or plastic state. The material has been variously thought to be an exudate from the blood, a pathologic secretion of the renal cells, and a product of epithelial degeneration. In the tubule it hardens into a cast which, when washed out by the urine, retains the shape of the tubule, and contains within its substance whatever structures and débris were lying free within the tubule or were loosely attached to its wall. If the tubule be small and has its usual lining of epithelium, the cast will be narrow; if it be large or entirely denuded of epithelium, the cast will be broad. *A cast, therefore, indicates the condition of the tubule in which it is formed, but does not necessarily indicate the condition of the kidney as a whole.* In any particular case of kidney disease several forms or even all may be found. Their number and the preponderance of certain forms will, as is shown later, furnish a clue to the nature of the pathologic process, but further than this one cannot go with certainty. One cannot rely upon the casts for accurate diagnosis of the histologic changes in the kidney.

At times during the course of a nephritis the urine is suddenly flooded with great numbers of tube-casts. Such "showers" may be of serious import, but are not necessarily so. In some cases they may result from a clearing out of the plugged renal tubules coincident with improvement and increased flow of urine.

The search for casts must be carefully made. The urine must be fresh, since hyaline casts soon dissolve when it becomes alkaline. It should be thoroughly centrifugalized. When the sediment is abundant, casts, being light structures, will be found near the top of the deposit. In cystitis, where casts may be entirely hidden by the pus, the bladder should be irrigated to remove as much of the pus as possible and the next urine examined. In order to prevent solution of the casts the urine, if alkaline, must be rendered acid by previous administration of boric acid or other drugs. Heavy sediments of urates, blood, or vaginal cells may likewise obscure casts and other important structures. The last can be avoided by catheterization. Urates can be dissolved by gently warming before centrifugalizing, care being taken not to heat enough to coagulate the albumin. The aluminum shield of the centrifuge tube may also be heated. Blood can be destroyed by centrifugalizing, pouring off the supernatant urine, filling the tube with water, adding a few drops of dilute acetic acid, mixing well, and again centrifugalizing; this process being repeated until the blood is completely decolorized. Too much acetic acid will dissolve hyaline casts.

In searching for casts the low-power objective should invariably be used, although a higher power may occasionally be desirable in studying details, as, for example, in distinguishing between an epithelial and a pus-cast. The casts are perhaps most frequently found near the edge of the cover-glass. Their cylindric shape can be best seen by slightly moving the cover-glass while observing them, or by pressing upon one edge of the cover with a needle, thus causing them to roll. This little manipulation should be practiced until it can be done satisfactorily. It will prove useful in many examinations.

Various methods of staining casts so as to render them more conspicuous have been proposed. They offer no special advantage to one who understands how to use the substage mechanism of his microscope. The "negative-staining" method is as good as any. It consists simply in adding a little India-ink to the drop of urine on the slide. Casts, cells, and other substances will stand out as colorless structures on a dark background. Some workers tinge the urine faintly with eosin or Lugol's solution, which are taken up by the casts.

(1) **Hyaline Casts.**—Typically these are colorless, homogeneous,

semitransparent, cylindric structures, with parallel sides and usually rounded ends. Not infrequently they are more opaque or show a few granules or an occasional cell or oil-globule, either adhering to them or contained within their substance. Generally they are straight or curved, less commonly convoluted (Fig. 75). Their

FIG. 65.—Hyaline and finely granular casts in urine; a "shower of casts." At the upper right is a mucous shred. A portion of an actual field (× 100).

length and breadth vary greatly; they are sometimes so long as to extend across several fields of a medium-power objective, but are usually much shorter; in breadth they vary from one to seven or eight times the diameter of a red blood-corpuscle (Figs. 6, 65, and 66).

FIG. 66.—Hyaline and finely granular casts enlarged from Fig. 65 (× 350).

Hyaline casts are the least significant of all the casts, and occur, usually in company with pale very finely granular casts, in many slight and transitory conditions. Small numbers are common following ether anesthesia, in fevers, after excessive exercise, and in congestions and irritations of the kidney. They are always present,

and are usually stained yellow when the urine contains much bile. While they are found in all organic diseases of the kidney, they are most important in chronic interstitial nephritis. Here they are seldom abundant, but their persistent presence is a significant sign of the disease. Small areas of chronic interstitial change are probably responsible for the few hyaline casts so frequently found in the urine of elderly persons.

Very broad hyaline casts commonly indicate complete desquamation of the tubular epithelium, such as occurs in the late stages of nephritis; or they may originate in relatively normal collecting tubules.

(2) **Waxy Casts.**—Like hyaline casts, these are homogeneous when typical, but frequently contain a few granules or an occasional

Fig. 67.—Waxy tube casts (× 350).

cell. They are much more opaque than the hyaline variety, and are usually shorter and broader, with irregular, broken ends, and sometimes appear to be segmented. They are grayish or colorless, and have a dull, waxy look, as if cut from paraffin (Figs. 67 and 90). They are sometimes composed of material which gives the amyloid reactions. All gradations between hyaline and waxy casts may be found, and doubtless many waxy casts are merely hyaline casts which have lain in the kidney tubules for a long time.

Waxy casts are found in most advanced cases of nephritis, where they are an unfavorable sign. They are perhaps most abundant in amyloid disease of the kidney, but are not distinctive of the disease as is sometimes stated.

(3) **Fibrinous Casts.**—Casts which resemble waxy casts, but have a distinctly yellow color, as if cut from beeswax, are often

seen in acute nephritis. They are called fibrinous casts, but the name is inappropriate, as they are not composed of fibrin. They are often classed with waxy casts, but should be distinguished, as their significance is much less serious. Their color is probably due to altered blood-pigment.

(4) **Granular Casts.**—These are merely hyaline casts in which numerous granules are embedded (Figs. 65, 66, 68, and 69).

Finely granular casts contain many fine granules, are usually shorter, broader, and more opaque than the hyaline variety, and are more conspicuous. Their color is grayish or pale yellow.

Coarsely granular casts contain larger granules and are darker in color than the finely granular, being often dark brown, owing to presence of altered blood-pigment. They are usually shorter and

FIG. 68.—Coarsely granular tube-casts (× 350).

FIG. 69.—Granular and fatty casts and two compound granule cells (Stengel).

more irregular in outline, and more frequently have irregularly broken ends.

(5) **Fatty Casts.**—Small droplets of fat may at times be seen in any variety of cast. Those in which the droplets are numerous are called fatty casts (Figs. 69 and 90). The fat-globules are not difficult to recognize. Staining with osmic acid or sudan III (p. 179) will remove any doubt as to their nature.

The granules and fat-droplets seen in casts are chiefly products of epithelial degeneration. Granular and fatty casts, therefore, always indicate partial or complete disintegration of the renal epithelium. The finely granular variety is the least significant, and may be found along with hyaline casts when the epithelium is only slightly and perhaps not seriously affected. Coarsely granu-

lar, and especially fatty casts, if present in considerable numbers, point toward a serious parenchymatous nephritis. Brown granular casts are most common in acute nephritis.

(6) **Casts Containing Organized Structures.**—Cells and other structures are frequently seen adherent to a cast or embedded within it. When numerous, they give name to the cast.

FIG. 70.—Tube-casts containing renal epithelial cells (× 350).

(*a*) *Epithelial casts* contain epithelial cells from the renal tubules. The cells vary in size, and are often flattened, oval, or elongated. They may be recognized as epithelial cells by irrigating with dilute acetic acid, which usually brings out the nucleus clearly. Epithelial

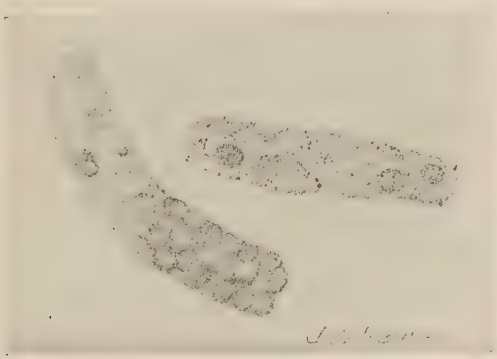


FIG. 71.—Tube-casts containing pus-corpuscles (× 350).

casts always imply desquamation of epithelium, which rarely occurs except in parenchymatous inflammations (Figs. 70, 89, and 90). When the cells are well preserved they point to acute nephritis.

(*b*) *Blood-casts* contain red blood-corpuscles, usually much degenerated (Figs. 72, 73, and 89). They always indicate hemorrhage

into the tubules, which is most common in acute nephritis or an acute exacerbation of a chronic nephritis.

(*c*) *Pus-casts* (Figs. 71 and 91), composed almost wholly of pus-corpuscles, are uncommon, and point to a suppurative process

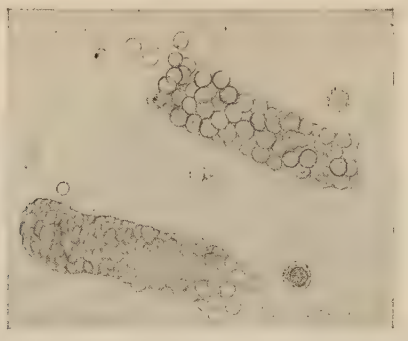


FIG. 72.—Two blood-casts, one containing a leukocyte; six free red blood-cells; and two renal epithelial cells. From the urine of a child with acute nephritis (× 300).

in the kidney, usually a pyelonephritis. Casts containing a few pus-corpuscles, either alone or in combination with epithelial or red blood-cells, are common, especially in acute nephritis. In these the pus-cells have no special significance.

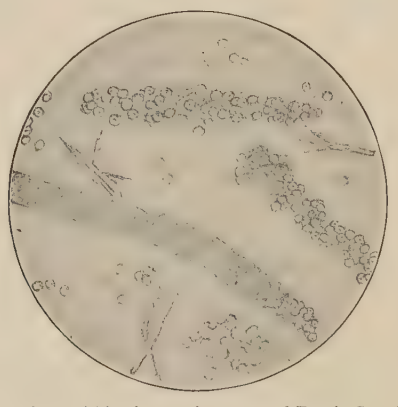


FIG. 73.—Red blood-corpuscles and blood-casts (courtesy of Dr. A. Scott) (obj. 4 mm.) (Boston).

(*d*) True *bacterial casts* are rare. They indicate a septic condition in the kidney. Bacteria may permeate a cast after the urine is voided.

Structures Likely to be Mistaken for Casts.—(1) **Mucous Threads.**—Mucus frequently appears in the form of long strands which slightly resemble hyaline casts (Fig. 74). They are, how-

ever, more ribbon-like, have less well-defined edges, and usually show faint longitudinal striations. Their ends taper to a point or are split or curled upon themselves, and are never evenly rounded, as is commonly the case with hyaline casts.

Such threads form a part of the nubecula of normal urine, and are especially abundant when calcium oxalate crystals are present.

FIG. 74.—Mucous threads in urine. These are often wrongly called cylindroids (× 350).

When there is an excess of mucus, as in irritations of the urinary tract, every field may be filled with an interlacing meshwork.

Mucous threads are microscopic, and should not be confused with urethral shreds or "gonorrheal threads," which are macro-

FIG. 75.—Four cylindroids and one convoluted hyaline cast (× 350).

scopic, 0.5 to 1 cm. long, and consist of a matrix of mucus in which many epithelial and pus-cells are embedded.

(2) **Cylindroids.**—This name is sometimes given to the mucous threads just described, but is more properly applied to certain peculiar structures more nearly allied to casts. They resemble hyaline casts in structure, but differ in that they taper to a slender

tail, which is often twisted or curled upon itself (Fig. 75). They frequently occur in the urine along with hyaline casts, especially in circulatory disturbances and irritations of the kidney, and have practically the same significance.

(3) **Masses of amorphous urates,** or **phosphates,** or **very small crystals** (Fig. 76), which accidentally take a cylindric form, or shreds of mucus covered with granules, closely resemble granular casts. The application of gentle heat or appropriate chemicals will serve to differentiate them. When urine contains both mucus and granules, large numbers of these "pseudocasts," all lying in the same direction, can be produced by slightly moving the cover-glass from side to side. It is possible—as in urate infarcts of infants—for urates to be molded into cylindric bodies within the renal tubules.

FIG. 76.—Two pseudocasts, one composed of calcium oxalate crystals, one of uric acid (× 300).

(4) **Hairs** and **fibers** of wool, cotton, and so forth. These could be mistaken for casts only by beginners. One can easily become familiar with their appearance by suspending them in water and examining with the microscope (Fig. 87).

(5) **Hyphæ of molds** are not infrequently mistaken for hyaline casts. Their higher degree of refraction, their jointed or branching structure, and the accompanying spores will differentiate them (Fig. 88).

2. Epithelial Cells.—A few cells from the epithelium of various parts of the urinary tract occur in every urine. A marked increase indicates some pathologic condition at the site of their origin. It is sometimes, but by no means always, possible to locate their probable source from their form, notably in the case of vaginal epithelium. One should, however, be extremely cautious about

making any definite statement as to the origin of any individual cell. Most cells are much altered from their original shape, and any may be so granular from degenerative changes that the nucleus is obscured. Many of them contain fat-globules. They may be divided into three groups. In reporting their presence at least the group to which they belong should be recorded.

(1) **Small round** or **polyhedral cells** are about the size of pus-corpuscles, or more frequently about one-third larger, with a single round nucleus. Such cells may come from the deeper layers of any part of the urinary tract. They are uncommon in normal urine. When they are polygonal in shape, rather dark in color, very granular, and contain a comparatively large nucleus

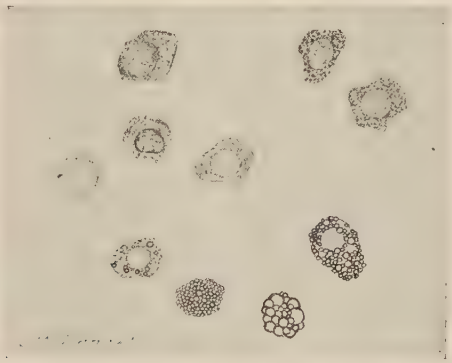


FIG. 77.—Renal epithelial cells from nephritic urine. The four cells below show different grades of fatty degeneration (× 475).

(Fig. 77), they probably come from the renal tubules, but their origin in the kidney is not proved unless they are found embedded in casts. In chronic passive congestion of the kidney, in renal infarction, and in hemochromatosis some of these cells may contain yellow granules of altered blood-pigment. They are analogous to the "heart-failure cells" of the sputum (p. 60). Renal cells are abundant in parenchymatous nephritis, especially the acute form. They are nearly always fatty—most markedly so in chronic parenchymatous nephritis, where their substance is sometimes wholly replaced by fat-droplets ("compound granule cells") (Figs. 69, 77, 89, and 90).

(2) The epithelial cells of the second group are larger than the small round cells just described, being two to four times the diam-

eter of a pus-corpuscle, and have various forms (Figs. 78 and 79). Commonly they are pear-shaped, spindle-shaped, or round, or have tail-like processes; and they are hence named pyriform, spindle, large round, or caudate cells, respectively. Each contains a round or oval nucleus, which generally stands out distinctly and is smaller in proportion to the size of the cell than is the nucleus of the typical renal cell. These cells are, for the most part, derived from the transitional epithelium which lines the bladder, ureters, and pelves of the kidneys; hence they may conveniently be grouped together as **transitional cells.** Cells of the same general type may, however, originate in the prostate and seminal vesicles, and, moreover, some

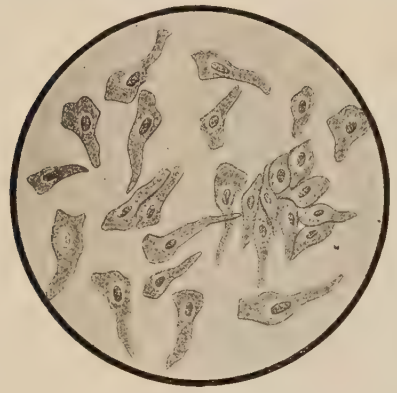


FIG. 78.—Caudate epithelial cells from pelvis of kidney (Jakob).

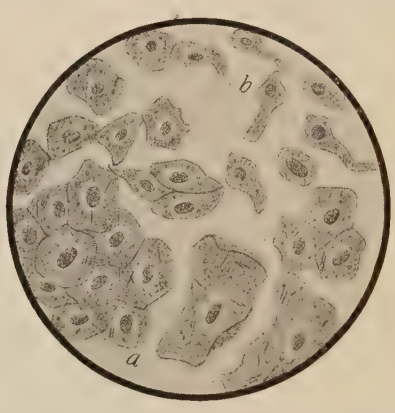


FIG. 79.—Epithelial cells from urethra and bladder: *a*, Squamous cells from superficial layers; *b*, irregular cells from deeper layers (Jakob).

of the superficial cells of the bladder are so thin and flat as to be more properly classed as squamous cells. Caudate forms apparently come most commonly from the pelvis of the kidney.

(3) **Squamous** or **pavement cells** are large flat cells, each with a small, distinct round or oval nucleus (Fig. 79, *a*). They are derived from the superficial layers of the urethra or vagina, and when desquamation is active appear in stratified masses. Squamous cells from the vagina are especially large, thin, and angular, and are sometimes rolled into cigar-like cylinders. Great numbers of these vaginal cells, together with pus-corpuscles, may be present when leukorrhea exists (Fig. 80). The most superficial of the cells lining the bladder are also thin and scale-like and may be classed

with the squamous cells. They are, however, generally less angular than are the vaginal cells.

3. Pus-corpuscles.—A very few leukocytes are present in normal urine, particularly when mucus is present. They are numerous only as a result of a pathologic process. The cells are then called pus-corpuscles and their presence constitutes *pyuria*. Although pus-corpuscles are less well preserved than are leukocytes and show more tendency to form small clumps, yet when only a few are present the line of distinction between them must be drawn arbitrarily and is best based upon the number present, although this depends largely upon the care used in preparing the slide. Students should be instructed to report "a very few pus-corpuscles" when they find an

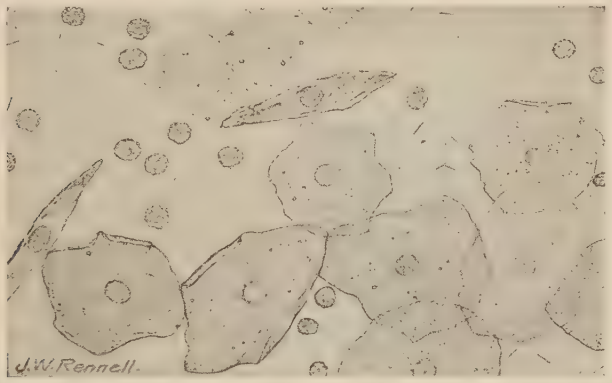


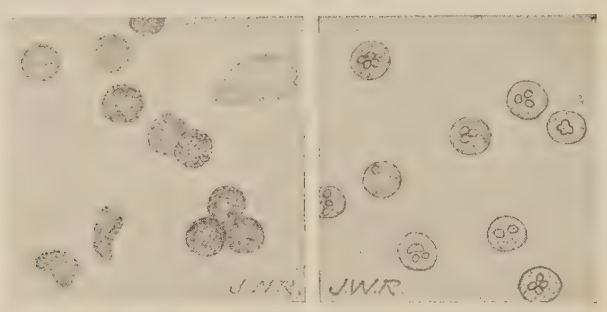
FIG. 80.—Squamous epithelial cells, pus-corpuscles, and bacteria in urine; vaginal contamination (× 300).

average of three or more of these cells to the field of the 6-mm. objective with 5× eye-piece. The great majority of pus-corpuscles are the polymorphonuclear leukocytes of the blood.

When at all abundant, pus adds an appreciable amount of albumin to the urine and forms a white sediment resembling amorphous phosphates macroscopically. Under the microscope the corpuscles appear as very granular spheric cells, about 10 to 12 μ in diameter or somewhat larger than red blood-corpuscles (Figs. 81 and 92). The granules are partly the normal neutrophilic granules, partly granular products of degeneration. In freshly voided urine many exhibit ameboid motion, assuming irregular outlines. Each pus-corpuscle contains one irregular nucleus or several small, rounded nuclei. The nuclei are obscured or entirely hidden by the

granules, but may be brought clearly into view by running a little dilute acetic acid under the cover-glass. This enables one to easily distinguish pus-corpuscles from small, round epithelial cells, which resemble them in size, but have a single, rather large, round nucleus. In moderately acid urine the cell structure is generally fairly well preserved. In very strongly acid urine the corpuscles may be shrunken and irregularly shaped, suggesting ameboid forms. When the urine is alkaline they are usually swollen, very granular, often ragged, and have a strong tendency to adhere in clumps; while in decomposing urine they are soon destroyed and converted into a gelatinous substance which gives the urine a mucilaginous consistence.

Pyuria indicates suppuration in some part of the urinary tract—urethritis, cystitis, pyelitis, and so forth—or may be due to



A B

FIG. 81.—Pus-corpuscles in urine: A, As ordinarily seen. At the lower left are two ameboid corpuscles. The large structure at the right is a bit of degenerated epithelium; B, when treated with acetic acid (× 475).

contamination from the vagina, in which case many vaginal epithelial cells will also be present. Of these conditions, chronic cystitis usually gives by far the greatest amount of pus. In general, the source of the pus can be determined only by the accompanying structures (epithelium, casts) or by the clinical signs. A considerable amount of pus, appearing suddenly, usually originates from a ruptured abscess.

A fairly accurate idea of the quantity of pus from day to day may be had by shaking the urine thoroughly and counting the number of corpuscles per cubic millimeter upon the blood-counting slide, but conditions—ingestion of water, and so forth—must be kept as nearly uniform as possible. A drop of the urine is placed directly upon the slide. Dilution is not necessary unless the cor-

puscles exceed 20,000 for each cubic millimeter. The urine must not be alkaline or the corpuscles will adhere in clumps. In cystitis the number of corpuscles runs from about 5000 for each cubic millimeter in mild cases to 100,000 or 150,000 in severe cases.

Pus always adds a certain amount of albumin to the urine, and it is often desirable to know whether the albumin present in a given specimen is due solely to pus. It has been estimated that 80,000 to 100,000 pus-corpuscles for each cubic millimeter add about 0.1 per cent. of albumin. If albumin is present in much greater proportion than this, the excess is probably derived from the kidney.

4. Red Blood-corpuscles.—Urine which contains blood is always albuminous. Very small amounts do not alter its macroscopic appearance. Larger amounts alter it considerably. Blood from the kidneys is generally intimately mixed with the urine and gives it a hazy, reddish, or brown "smoky" color. When from the lower urinary tract, it is not so intimately mixed and settles more quickly to the bottom, the color is brighter, and small clots are often present. A further clue to the site of the bleeding may sometimes be gained by having the patient void his urine in three separate portions. If the blood be chiefly in the first portion, the bleeding point is probably in the urethra; if in the last, it is probably in the bladder. If the blood is uniformly mixed in all three portions, it probably comes from the kidney or ureter. Microscopically the presence of tube-casts or of considerable numbers of epithelial cells of the renal type would be suggestive, while the presence of blood-casts would, of course, point definitely to hemorrhage into the kidney tubules.

Red blood-corpuscles are not usually difficult to recognize with the microscope. When very fresh they have a normal appearance, being yellowish disks of uniform size. They are apt to be swollen in dilute and crenated in concentrated urines. When they have been in the urine any considerable time their hemoglobin may be dissolved out, and they then appear as faint colorless circles or "shadow cells," and are more difficult to see (Fig. 82; also Figs. 72, 73, and 89). The shadow cells are not always uniform in size, and, while usually circular, may be oval, pear-shaped, or irregular in outline. The microscopic findings may be corroborated by chemical tests for hemoglobin, although the microscope may show a few red

corpuscles when the chemical tests are negative. When the blood-cells are very numerous, they are often accompanied by yellowish shreds of fibrin of various sizes.

When not due to contamination from menstrual discharge, blood in the urine, or *hematuria*, is always pathologic, and usually, although by no means always, of serious import. A few red blood-corpuscles may be found after strenuous exercise. Blood comes from the *kidney tubules* in severe hyperemia, in acute nephritis and exacerbations of chronic nephritis, and in renal tuberculosis and malignant disease. Renal hematuria may also be a manifestation of the "hemorrhagic diseases"; and an "idiopathic hematuria," probably of nervous origin, has been described. Usually, however, the term "idiopathic hematuria" covers a failure in diagnosis. The

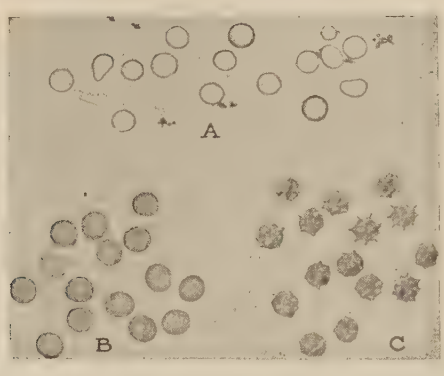


FIG. 82.—Red blood-corpuscles in urine: *A*, Shadow cells from a case of nephritis; *B*, fresh red corpuscles; *C*, crenated corpuscles in a urine of high specific gravity (× 475).

urine of healthy infants frequently contains red blood-corpuscles for weeks at a time. This has been attributed to slight toxic injury to the kidneys, which are particularly sensitive in infancy. Blood comes from the *pelvis of the kidney* in the presence of renal calculus, of which hematuria is the classical and most constant symptom. The bleeding in this condition is usually intermittent, small in amount, and accompanied by a little pus and perhaps crystals of the substance forming the stone. Considerable hemorrhages from the *bladder* may occur in vesical calculus, tuberculosis, and new growths. Small amounts of blood generally accompany acute cystitis. In Africa the presence of *Schistosoma hæmatobium* in the veins of the bladder is a common cause of hemorrhage (Egyptian hematuria).

5. Spermatozoa are generally present in the urine of men after nocturnal emissions, after epileptic convulsions, and in spermatorrhea. They may be found in the urine of both sexes following coitus. They are easily recognized from their characteristic structure (Fig. 83). The 4-mm. objective should be used, with subdued light and careful focusing.

6. Bacteria.—Under normal conditions urine is free from bacteria in the bladder, but becomes contaminated in passing through the urethra. Various non-pathogenic bacteria, notably *Micrococcus ureæ*, which forms chains like those of the streptococci, are present in old or decomposing urine. They are easily seen with the 4-mm. objective in the routine microscopic examination, but ordinarily no attempt is made to identify them. They produce a cloudiness which will not clear upon filtration through paper.

FIG. 83.—Spermatozoa in urine. A pus-corpuscle and a transitional epithelial cell are also shown (× 475).

In many infectious diseases the specific bacteria may be eliminated in the urine without producing any local lesion. Typhoid bacilli are present in the urine in about 30 per cent. of the cases, and have been known to persist for months and even years after the attack.

Tubercle bacilli are nearly always present in the urine when tuberculosis exists in any part of the urinary tract, and are often present in general miliary tuberculosis, but may be difficult to find, especially when the urine contains little or no pus.

In local non-tuberculous infections of the urinary tract, notably in acute and chronic pyelitis and cystitis, a variety of bacteria have been found. In more than half of the cases the colon bacillus is present alone or in company with others. Next most frequent are

the staphylococci. *Bacillus proteus vulgaris*, *Bacillus pyocyaneus*, streptococci, and others have also been encountered.

Detection of Tubercle Bacilli in Urine.—In order to avoid the smegna bacillus the urine should be obtained aseptically by catheter after careful cleansing of the parts, or by having the patient void urine in three portions, only the last being used for the examination.

1. Concentrate the tubercle bacilli into a small amount of sediment. This may be done by simply centrifugalizing thoroughly at high speed, or, better, by the method of Petroff, as follows:

(*a*) Acidify 100 c.c. of the urine with 30 per cent. acetic acid, add 2 c.c. of 5 per cent. tannic acid solution, and mix.

(*b*) Place in the refrigerator for twenty-four hours.

(*c*) Centrifugalize thoroughly at high speed, pipet off the supernatant fluid, and redissolve the sediment with dilute acetic acid.

(*d*) Centrifugalize thoroughly once more.

2. Make thin smears of the sediment, adding a little egg-albumen if necessary to make the smear adhere to the glass; dry, preferably in the incubator, for three hours, and fix in the usual way.

3. Stain thoroughly with carbolfuchsin in the usual way (p. 65).

4. Wash in water, and then in 5 per cent. nitric acid, until only a faint pink color remains.

5. Wash in water.

6. Soak in alcohol fifteen minutes or longer. This decolorizes the smegma bacillus (p. 69), which is often present in the urine, and might easily be mistaken for the tubercle bacillus. Some strains of the smegma bacillus are very resistant to alcohol. It is therefore best to avoid it altogether by examining only catheterized specimens, in which case this step may be omitted.

7. Wash in water.

8. Apply Loeffler's methylene-blue solution for a few seconds. The blue stain should be light.

9. Rinse in water, dry between filter-papers, and examine with the oil-immersion objective.

A careful search of several smears may be necessary to find the bacilli. They usually lie in clusters (Plate IV). Failure to find them in suspicious cases should be followed by inoculation of guinea-pigs; this is the court of last appeal, and must also be sometimes resorted to in order to exclude the smegma bacillus.

Detection of Gonococci in Urine.—In acute and chronic gonorrhea gonococci can sometimes be found within pus-cells in the sediment, but more commonly in the "gonorrheal threads" or "floaters," which are

PLATE IV

Tubercle bacilli in urinary sediment; × 800 (Ogden).

described on page 191. In themselves these threads are by no means diagnostic of gonorrhea. They are most common in the morning or after massage of the prostate. The floater is fished out, spread upon a slide, dried, and fixed by heat. It is then stained and searched for gonococci as described on page 524. Recognition of the gonococcus in isolated pus-cells of the sediment is difficult since these cells are usually much shrunken. The smears should be thin and quickly dried.

Method for Urine Cultures.—The tubercle bacillus and the gonococcus are generally sought by staining methods as above described. Others can be found and identified only by culture.

The urine must be collected in a sterile test-tube or bottle with every precaution to avoid contamination. In the case of females this necessitates catheterization. In males it will suffice to wash the glans penis and margins of the urethral orifice with green soap and water followed

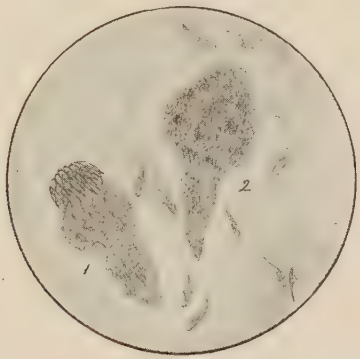


FIG. 84.—1, Scolex of *Tænia echinococcus*, showing crown of hooklets; 2, scolex and detached hooklets (obj. 4 mm.) (Boston.)

by mercuric chlorid in 1 : 1000 solution. The urine is then voided and most of it is allowed to escape, only the latter portion being saved. A part of this should be thoroughly centrifugalized (adding alcohol if the specific gravity is high), and stained smears from the sediment should be studied in order to gain approximate knowledge of the number and kind of bacteria present. From the remaining portion of the urine cultures should be made upon plain agar and Endo's medium, and, if streptococci are expected, also upon blood-agar or ascitic-fluid-agar. The amount of urine to plant in order to secure isolated colonies must be judged from the number of bacteria found in the preliminary microscopic examination. It may be necessary to dilute the urine with several times its volume of nutrient broth or physiologic salt solution before spreading it upon the media. Upon the other hand, when bacteria are very scarce it may be necessary to use the concentrated sediment after

long centrifugation, but this, of course, should not be used if alcohol has been added to favor sedimentation.

7. Animal parasites are rare in the urine. Hooklets and scolices of *Tænia echinococcus* (Fig. 84) and larvæ of filariæ have been met. In Africa the ova, and even adults, of *Schistosoma hæmatobium* are common, accompanying "Egyptian hematuria." *Trichomonas hominis* is a not uncommon contamination, usually reaching the urine from the vagina or the rectum. This and other protozoa may be mistaken for spermatozoa by the inexperienced.

A worm which is especially interesting is *Anguillula aceti*, the "vinegar eel." This is generally present in the sediment of table vinegar, and may reach the urine through use of vinegar in vaginal

FIG. 85.—Larva of "vinegar eel" in urine, from contamination; length, 340 μ; width, 15 μ. An epithelial cell from bladder and three leukocytes are also shown.

douches, or through contamination of the bottle in which the urine is contained. It has been mistaken for *Strongyloides stercoralis* and for the larval filaria. It somewhat resembles the former in both adult and larval stages. The young larvæ have about the same length as the larvæ of *Filaria bancrofti*, but are nearly twice as broad, and the intestinal canal is comparatively easily seen (compare Figs. 85 and 230).

For fuller descriptions of these parasites the reader is referred to Chapter VI.

C. Extraneous Structures

The laboratory worker must familiarize himself with the microscopic appearance of the more common of the numerous structures which may be present from accidental contamination (Fig. 87).

Yeast-cells are smooth, colorless, highly refractive, spheric or

ovoid cells. They sometimes reach the size of a leukocyte, but are generally smaller (Fig. 86). They are often mistaken by the in-

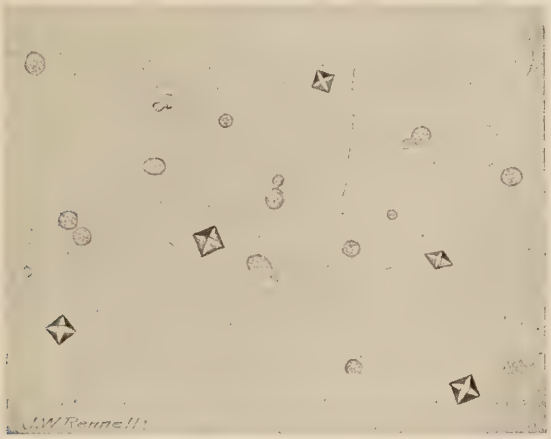


FIG. 86.—Yeasts and calcium oxalate crystals in a urine which had been preserved for two weeks with boric acid. Note the budding forms which are characteristic of the yeasts (× 450).

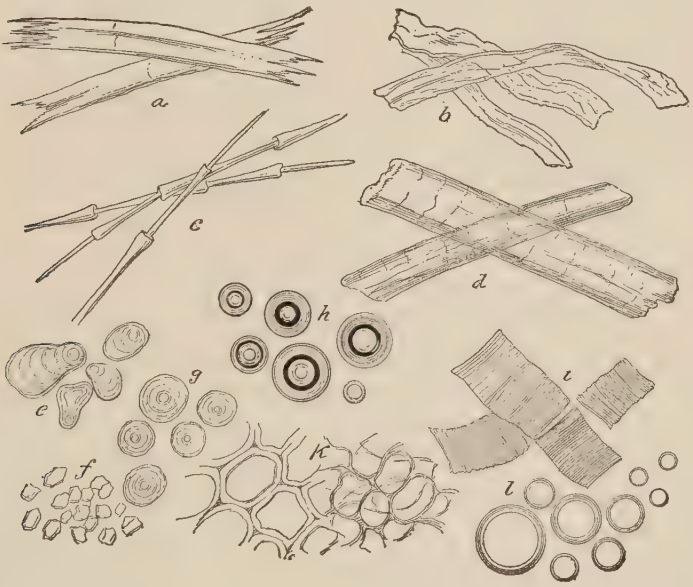


FIG. 87.—Extraneous matters found in urine: *a*, Flax-fibers; *b*, cotton-fibers; *c*, feathers; *d*, hairs; *e*, potato-starch granules; *f*, rice-starch granules; *g*, wheat-starch granules; *h*, air-bubbles; *i*, muscular tissue; *k*, vegetable tissue; *l*, oil-globules.

experienced for red blood-corpuscles, and more rarely for fat-droplets or the spheric crystals of calcium oxalate, but are dis-

tinguished by the facts that they are usually ovoid and not of uniform size; that they tend to adhere in short chains; that small buds may often be seen adhering to the larger cells; and that they do not give the hemoglobin test, are not stained by osmic acid or sudan III, but are colored brown by Lugol's solution, and are insoluble in acids and alkalies. Yeast-cells multiply rapidly in diabetic urine, and may reach the bladder and multiply there.

Mold fungi (Fig. 88) are characterized by refractive, jointed, or branched rods (hyphæ), often arranged in a network, and by highly refractive spheric or ovoid spores. They are common in urine which has stood exposed to the air. Not infrequently a spore with a short hypha growing from it is reported as a spermatozoön. The spores sometimes tend to form short chains.

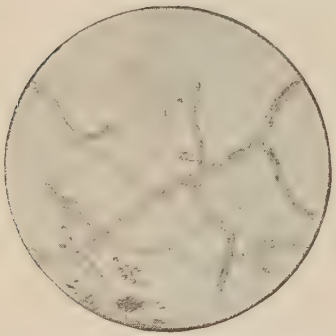


Fig. 88.—Aspergillus from urine (Boston).

Fibers of wool, cotton, linen, or silk, often colored, derived from towels, the clothing of the patient, or the dust in the air, are present in almost every urine. They are best identified by comparison with known fibers which are placed in a drop of water on a slide and covered. **Fat-droplets** are most frequently derived from unclean bottles or oiled catheters. **Starch-granules** may reach the urine from towels, the clothing, or dusting-powders. They are recognized by their concentric striations, which, however, are sometimes difficult to see, and by the blue color which they take when treated with weak iodin solution. **Lycopodium granules** (Fig. 12) may also reach the urine from dusting-powders. They might be mistaken for the ova of parasites. **Bubbles of air** (Fig. 87, *h*) are often confusing to beginners, but are easily recognized after once being seen. Another common source of confusion, which does not seem to be well enough recognized, is **pollen** from flowers which have been kept in the sick room. The pollen granules differ in the different species, but are generally clean cut, more or less rounded, light yellow to brown bodies, and bear little resemblance to the ova for which they are sometimes reported. **Diatoms** from the tap-water are not infrequently encountered, and certain of the elongated forms have been mistaken for tube-casts.

Scratches and **flaws** in the glass of slide or cover are often most assiduously studied by beginners, and are not infrequently reported as rare crystals, tube-casts, or even worms. **Dirt** upon the top of the cover (especially when this is taken directly from the original box without cleaning) is likewise a common source of confusion. It often takes the form of crystals which, because they are more prominent than the structures in the urine beneath the cover, receive the student's whole attention. Fibers of **muscle** (Figs. 87, *i*, and 183) and other particles which are evidently of fecal origin are usually the result of accidental contamination, but may rarely be present in catheterized specimens. They then indicate rectovesical fistula. A suspicion of fecal contamination may be confirmed by a strong urobilin test.

V. THE URINE IN DISEASE

In this section the characteristics of the urine in those diseases which produce distinctive urinary changes will be briefly reviewed.

1. Acute Congestion of the Kidneys.—This regularly occurs as an early stage of acute nephritis, but more frequently occurs independently as a result of the temporary irritation of toxins, certain drugs, and so forth. The urine is decreased in volume, highly colored, strongly acid. Albumin is always present, varying from traces to considerable amounts, depending upon the severity of the case. The sediment shows a few hyaline and finely granular casts and occasional red blood-corpuscles. In severe acute congestion the urine approaches that of acute nephritis, since there is, in fact, no sharp line to be drawn between the two conditions.

2. Chronic Passive Congestion of the Kidneys.—This occurs most commonly as a part of general venous stasis due to cardiac decompensation. The volume of urine is somewhat low and the color and specific gravity high. As a rule, albumin is present in small amount only; in very marked congestion it becomes more abundant. As the liver is generally damaged in cases of long-continued venous stasis, moderate amounts of urobilinogen may be found in the urine. The sediment contains a few hyaline and finely granular casts; and a few red blood-corpuscles and renal epithelial cells may occasionally be found. Estimation of blood urea fails to show any appreciable amount of nitrogen retention in cases of passive hyperemia, whereas the phenolsulphonephthalein test indicates

a marked degree of kidney insufficiency which improves when the congestion is relieved.

3. Nephritis.—The various degenerative and inflammatory conditions which are grouped under the name of nephritis occupy an extremely important place in medicine and deserve somewhat extended discussion. Owing to lack of correlation between clinical observations and the microscopic structure of kidneys removed at autopsy there is no classification which is satisfactory alike to clinician and pathologist. The forms which are generally given consideration in clinical work are few in number, namely, acute nephritis; chronic diffuse (parenchymatous) nephritis; chronic interstitial nephritis, which includes the arteriosclerotic type; and pyelonephritis, which represents an upward extension of an inflammation already established in the pelvis of the kidney. In acute diffuse nephritis the injury may be borne chiefly by the glomeruli, as in the nephritis of scarlet fever, or by the epithelium of the convoluted tubules, as in poisoning by mercuric chlorid. It should therefore be possible to distinguish an acute glomerulonephritis and an acute tubular form, but the distinction may be difficult or impossible in practice except by consideration of etiology. In a sense, except perhaps at the very outset, nephritis is always diffuse, since glomeruli, tubules, and interstitial tissue are all involved to some extent, regardless of which of them has sustained the primary and chief injury. In the course of a chronic nephritis now one of these, now another, may dominate the clinical and pathologic picture.

There is a tendency at present to discard the anatomic classification altogether. Some, following Christian, divide nephritis into acute and chronic, with subdivisions of the latter based upon the presence or absence of edema or vascular hypertension.

In view of the unfortunate tendency of many physicians to rely solely upon the laboratory report for a diagnosis of nephritis, the fact will bear emphasis that here, as in most conditions, the laboratory observations furnish only a part of the data necessary for a diagnosis. Even with every available aid the diagnosis of nephritis may be difficult and the designation of the exact type impossible.

In all forms of nephritis the urine contains **albumin** and **tube-casts.** These are the classical signs, and they remain the most delicate indicators of pathologic changes in the kidneys, although they tell little of its nature, extent, and seriousness. The amount

of albumin is extremely variable, depending upon the form and severity of the disease. It ranges from traces so small as to be overlooked in a careless examination in many cases of chronic interstitial nephritis, to as high as 3 or 4 per cent. in exceptional cases of chronic parenchymatous nephritis. Urine containing more than 2 per cent. of albumin solidifies upon boiling. Upon the other hand, as has already been emphasized, albumin may be present even in considerable amount in many conditions other than nephritis—a fact not well enough appreciated by the average practitioner. The presence of tube-casts has also been popularly ascribed greater significance than is warranted, especially in differentiating

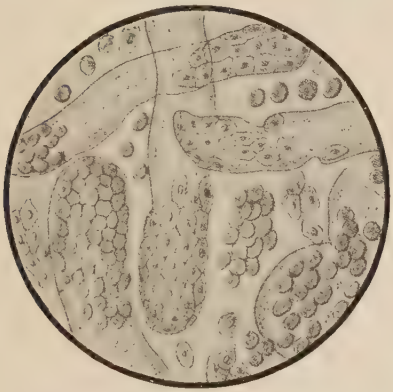


FIG. 89.—Sediment from acute hemorrhagic nephritis: Red blood-corpuscles; leukocytes; renal cells not fattily degenerated; epithelial and blood-casts (Jakob).

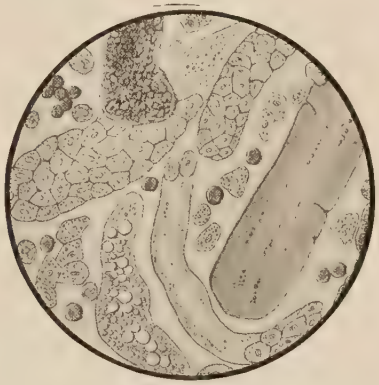


FIG. 90.—Sediment from chronic parenchymatous nephritis: Hyaline (with cells attached), waxy, brown granular, fatty, and epithelial casts; fattily degenerated renal cells, and a few white and red blood-corpuscles (Jakob).

the several forms of nephritis. A few tube-casts of the hyaline and finely granular varieties are often encountered in routine examinations, and may point to nothing more than some slight temporary irritation or circulatory disturbance in the kidney or the sclerotic change incident to old age. Nevertheless, the presence of casts always indicates some disturbance in the kidneys, and *their continued presence should arouse strong suspicion of nephritis unless other adequate cause is obvious.* Much more significant of nephritis are coarsely granular, fatty, epithelial, and blood-casts.

In addition to casts, the microscope may reveal, according to the variety of nephritis, red blood-corpuscles, usually in the form

of "shadow cells"; renal epithelial cells, more or less degenerated; and small or moderate numbers of pus-corpuscles.

An important feature of nephritis is the failure of the kidneys adequately to excrete **normal solids,** which results in an accumulation of these substances in the body. Chief interest attaches to sodium chlorid and the nitrogenous waste products typified by urea. In some cases urea only is retained, in others sodium chlorid, and in still others both these substances. In the case of urea, retention is best measured by the exact methods of blood chemistry and is discussed at another place. Sodium chlorid retention is generally associated with edema, and is most typically seen in chronic diffuse (parenchymatous) nephritis in which edema is a common symptom. During a period of elimination of edema the sodium chlorid of the urine is strikingly increased. The total volume of the urine likewise demands attention when nephritis is suspected, being diminished in well-defined cases of some forms and much increased in others.

The more characteristic features of the urine in the various types of nephritis are indicated in the table on page 209, and need not be repeated here, but the data there set down cannot be relied upon implicitly. They show what may be expected in typical cases. The findings are often subject to marked variations at different periods in the course of the same case. For example, even in typical chronic interstitial nephritis there may at times occur marked epithelial degeneration in small groups of tubules, and these may supply the urine with degenerated epithelial cells, coarsely granular, epithelial or fatty casts, and other structures commonly regarded as characteristic of chronic parenchymatous nephritis.

Perhaps the most useful of all laboratory methods which aid in recognizing the disease and in following the progress of individual cases when diagnosed are the various **functional tests,** which represent the chief advance in the study of nephritis within the past few years. They should, however, be used with full appreciation that some of the functions may be disturbed, while others remain unaltered, and that the efficiency of the kidneys may be markedly impaired by various extrarenal conditions, the chief of which are listed on page 95. Upon the other hand, owing to the remarkable reserve power of the kidneys, their function is some-

THE URINE IN NEPHRITIS

	Physical.	Chemic.	Microscopic.
Acute nephritis.	Quantity diminished, often very greatly. Color dark; may be red or smoky. Specific gravity, 1.020 to 1.030.	Urea and chlorids low. Much albumin: up to 1.5 per cent. Reaction acid.	Sediment abundant, red or brown. Many casts, chiefly granular, blood and epithelial varieties; fatty casts in convalescence. Red blood-cells abundant. Numerous renal epithelial cells. Few pus-corpuscles.
Chronic diffuse (parenchymatous) nephritis. (Large white kidney.)	Quantity usually diminished. Color variable, often pale and hazy. Specific gravity, 1.010 to 1.020.	Urea and chlorids variable. Largest amounts of albumin: up to 3 per cent. Reaction acid.	Sediment rather abundant. Many casts of all varieties: fatty casts and casts of degenerated epithelium most characteristic. Blood present in traces: abundant only in acute exacerbations. Numerous fattily degenerated renal epithelial cells, often free globules of fat, and a few leukocytes.
Chronic interstitial nephritis. (Contracted kidney.)	Quantity markedly increased, especially at night. Color pale, clear. Specific gravity, 1.005 to 1.015.	Urea and chlorids low in advanced cases. Albumin present in traces (often overlooked), increasing in late stages. Reaction acid.	Sediment very slight. Few narrow hyaline and finely granular casts. No blood except in acute exacerbations. Very few renal cells. Uric acid and calcium-oxalate crystals common.
Pyelonephritis.	Quantity often increased and color pale; slightly or moderately cloudy. Specific gravity normal or low.	Urea and chlorids low in advanced cases. Albumin more abundant than can be explained by pus. Reaction acid.	Variable amount of pus. Many varieties of casts, pus casts most characteristic. Renal and pelvic epithelial cells. Red blood-corpuscles occasionally.
Amyloid degeneration of kidney.	Quantity normal or moderately increased. Color pale, clear. Specific gravity, 1.012 to 1.018.	Slight decrease of urea and chlorids. Variable amounts of albumin and globulin.	Sediment slight. Moderate number of hyaline, finely granular, and sometimes waxy casts.

times not appreciably disturbed by actual organic disease until the damage is considerable. Indeed, it is well known that in some early cases of nephritis the functional activity of the kidneys may be temporarily exalted, owing probably to irritation of the uninjured portions. Even after the disease is well established there may be marked divergence between the functional derangement and the recognizable anatomic lesions. This is particularly true in acute nephritis and in the earlier stages of chronic diffuse (parenchymatous) nephritis.

Of all the means of estimating renal function, the *phenolsulphonephthalein test* has found widest acceptance, partly because it has been so long in use that its reliability is well established, partly because it is easily carried out in office practice without need of special apparatus or unusual skill.

As has already been indicated, the normal kidneys excrete 60 to 75 per cent. of the dye in the two-hour period allowed for the test. A fall to 40 per cent. indicates impaired function and demands serious attention, although it does not necessarily mean nephritis. Reduction to 20 or 30 per cent. is the rule in well-marked cases of nephritis; while excretion of less than 10 per cent. may usually be interpreted as forecasting an early fatal termination. Exceptionally, deaths from uremia have occurred when the output was as high as 40 per cent. This, like most of the functional tests, finds its greatest usefulness in the study of chronic interstitial nephritis, the form of kidney disease which is most often met in general practice and in which the urinary changes are least striking. Here the phenolsulphonephthalein output appears to be a very satisfactory index to the extent of the pathologic changes in the kidneys. In some cases a greatly lowered output may be the most striking evidence of a very serious kidney condition.

Despite its unquestioned usefulness in the diagnosis and prognosis of nephritis, the test has certain limitations which are too frequently overlooked in practical work. The chief of these, some of which are shared by the other functional tests, are:

(*a*) Entirely normal or even increased excretion is sometimes noted in acute nephritis, especially in acute glomerulonephritis.

(*b*) In certain other cases of acute nephritis extremely low excretion, reduced almost to zero, which in chronic nephritis would

point to an inevitable early fatal outcome, may exceptionally be followed by complete recovery.

(*c*) For a short period at the outset of a chronic nephritis there may be exalted renal activity, with an output of 70 to 85 per cent.

(*d*) Low excretion, exceptionally as low as 10 to 15 per cent., is the rule in cardiac decompensation with chronic passive congestion of the kidneys, and this rises rapidly when the congestion is relieved by improved heart action.

(*e*) In chronic diffuse (parenchymatous) nephritis, although the output is significant, it sometimes presents inexplicable variations.

The *test-meal for renal function* first advocated in this country by Mosenthal appears to be especially useful for the detection of very early kidney disease, since it may give definite evidence of renal disturbance at a time when the other functional tests still indicate normal or exalted function. The method is simple enough to be readily carried out in private practice. The principle of the test and the exact procedure, together with the findings under normal conditions, are described on page 101. In chronic interstitial nephritis the findings after the test diet are remarkably constant. The earliest sign is usually nocturnal polyuria—night urine above 600 or 700 c.c.—and this may be noted when there are no other evident signs, or only a trace of albumin and a few hyaline casts in the urine. This is followed somewhat later by a lowering and a fixation of the specific gravity, both of which changes gradually become more marked as the disease advances. In advanced cases the specific gravity of the two-hour specimens is fixed at about 1.010 or lower, with maximum variation of only 1 to 3 points. In chronic diffuse (parenchymatous) nephritis, the urinary findings after the test-meal are variable.

The simple concentration and dilution tests mentioned on page 103 are also very valuable in detecting chronic nephritis.

As has been stated in previous pages, the *degree of nitrogen retention* in the blood has come to be relied upon as an index of the functional efficiency of the kidneys. This is of great value in the differential diagnosis of well-established cases, and is especially useful in prognosis since it serves as a direct measure of the tendency to uremia, even though the true nature of uremia and the particular substance which may be responsible for it are alike unknown. The methods used for estimation of nitrogen retention and

the normal values have been discussed on page 102; while the figures to be expected in progressive grades of kidney disturbance are given in the table on page 97. While a non-protein nitrogen concentration above 90 mg. for each 100 c.c. of blood or urea nitrogen above 65 mg. is a reliable sign of a grave outlook, the figures generally accepted as indicating slight grades of renal insufficiency (for example, non-protein nitrogen 30 to 45, or urea nitrogen of 16 to 27 mg. for each 100 c.c. of blood) cannot be relied upon unreservedly, since, owing chiefly to excess of protein in the diet, they may exceptionally be equaled when the kidneys are normal. Upon the other hand, since the kidneys have a capacity for the excretion of urea far in excess of normal demands they may be able to prevent accumulation of nitrogen even when seriously damaged.

In chronic passive congestion of the kidneys the blood urea and non-protein nitrogen undergo little or no change. Their estimation, therefore, taken in conjunction with the phenolsulphonephthalein test, which gives low values in this condition, may be of great service in cardiorenal conditions as a means of separating primary renal disease with secondary cardiovascular change from secondary renal disturbances resulting from passive congestion due to a poorly compensated heart lesion.

The principal substances concerned in "nitrogen retention" are uric acid, urea, and creatinin. It is believed by some, following the work of Myers, Fine, and Lough, that estimation of these substances separately gives the best insight into the renal condition. According to their view there is a definite order in which the substances are retained. Thus, damage to the kidneys is first manifested functionally by retention of uric acid; and increase of this substance in the blood may therefore be the earliest sign of kidney disease, especially of chronic interstitial nephritis. Of course, other sources of uric acid accumulation, such as gout, must be excluded. As the disease progresses urea also begins to accumulate; and when the function is markedly depressed all three substances are retained. According to Myers and Lough, the creatinin concentration in the blood is the surest guide to a serious prognosis, any appreciable retention indicating a grave disorder of the renal function, and a rise to 5 mg. for each 100 c.c. of blood warranting a hopeless prognosis. Not all, however, have found the creatinin values so significant. In this connection it is necessary to recognize the work of Behre

and Benedict, which indicates that there is no creatinin in the blood, and that the substance which has hitherto been thought to be creatinin is some unknown substance which reacts in a similar manner in the methods now in use. This does not necessarily overthrow the conclusions regarding the value of the determinations.

The **blood-picture** in nephritis may for convenience be mentioned here. In chronic interstitial nephritis the blood usually exhibits the changes characteristic of a moderate secondary anemia, which may become fairly marked as the disease progresses. If cardiac decompensation supervenes, the red corpuscles and hemoglobin tend to rise to normal or even above. In chronic diffuse (parenchymatous) nephritis the anemia is much more striking, red corpuscles often falling to 2,500,000 and hemoglobin sinking to a corresponding level. Brown considers this of grave prognostic significance, indicating injury to the bone marrow. There may be moderate polymorphonuclear leukcytosis late in the disease.

4. Amyloid Disease of the Kidneys.—Here the pathologic picture is that of a chronic nephritis plus a deposit of amyloid material in the glomerular tufts and interstitial tissue. The urine is abundant, of low specific gravity, and fairly rich in albumin and globulin. Urinary solids are not generally much decreased unless the disease is far advanced. Casts appear in variable numbers. These are chiefly hyaline; but granular, waxy, and occasional fatty casts also occur. The waxy casts are regarded as most typical, although, contrary to an old belief, they are rarely composed of true amyloid material.

5. Renal Tuberculosis.—In early cases the urine may be practically normal in appearance, but is more frequently pale and somewhat cloudy from the presence of a small amount of pus. Pyuria with no bacterial growth in ordinary cultures is strongly suggestive of tuberculosis. The volume may not be affected, but is apt to be increased, the reaction is acid, and there are traces of albumin and a few renal cells. In advanced cases, or those in which the pelvis of the kidney is involved in the tuberculous process, the urine is usually pale, cloudy, and alkaline, has an offensive odor, and is irritating to the bladder. In such cases albumin and pus are always present, though frequently not abundant. The pus is generally intimately mixed with the urine and does not settle so

quickly as does the pus of cystitis. Casts, though present, are seldom abundant, and are obscured by the pus. Traces of blood are common. Tubercle bacilli are nearly always present even when the pus is extremely slight, and their detection is essential for the diagnosis. In most cases they can be found by appropriate staining of the sediment (p. 200). The possibility of confusion with the smegma bacillus must, of course, be kept in mind. In other cases inoculation of guinea-pigs will be necessary. This method of detecting tubercle bacilli, which is described on page 200, is so simple and so trustworthy that it should be resorted to in all doubtful cases.

Temporary or permanent occlusion of the ureter on the diseased side, with consequent change in the urinary findings, occurs, according to Braasch, in about 10 per cent. of cases.

6. Malignant Tumors of the Kidney.—Hematuria, intermittent or constant, moderate or very marked, is the only urinary sign which occurs with any degree of regularity. It is observed at some time in the course of practically all cases, and in probably three-quarters of them it is the first definite symptom.

7. Renal Calculus.—The urine is usually somewhat concentrated, with high color and strongly acid reaction. Small amounts of albumin and a few casts may be present as a result of kidney irritation. Blood is frequently present, especially in the daytime and after severe exercise. Crystals of the substance composing the calculus—uric acid, calcium oxalate, cystin—may often be found in the freshly voided urine. The presence of a calculus generally produces pyelitis, and variable amounts of pus then appear, the urine remaining acid in reaction.

8. Pyelitis.—In pyelitis the urine is slightly acid, and contains a small or moderate amount of pus, together with many spindle and caudate epithelial cells. These findings may be intermittent, owing to occasional blocking of the ureter on the diseased side. Pus-casts and also other forms may appear when the process extends up into the kidney tubules as is usually the case (Fig. 91). Albumin is always present, and its amount, in proportion to the amount of pus, is decidedly greater than is found in cystitis. This fact is of much value in differential diagnosis. Even when pus is scanty albumin is rarely under 0.15 per cent., which is the maximum amount found in cystitis with abundant pus.

Bacteriologic studies reveal the colon bacillus in about half of the cases. Staphylococci, *Bacillus proteus vulgaris*, and others have been found.

9. Cystitis.—In acute and subacute cases and in many chronic cases of moderate severity the urine is acid and contains a variable amount of pus, with many epithelial cells from the bladder—chiefly large round and pyriform cells. Red blood-corpuscles are often numerous. Albumin is present in small amount—less than 0.15 per cent. Of the micro-organisms, the colon bacillus is most frequently found in these cases.

In neglected chronic cases and those due to prostatic obstruction the urine is generally alkaline. It is pale and cloudy from the

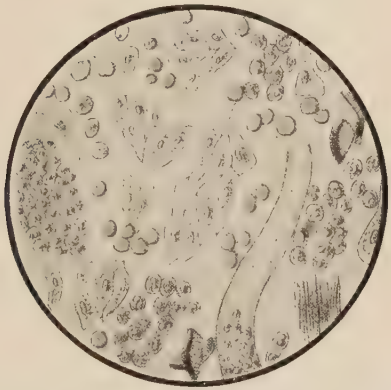


FIG. 91.—Sediment from calculous pyelitis: Numerous pus-corpuscles, red blood-corpuscles, and caudate and irregular epithelial cells; a combination of hyaline and pus-casts and a few uric-acid crystals (Jakob).

presence of pus, which is abundant and settles readily into a viscid sediment. The sediment usually contains abundant amorphous phosphates and crystals of triple phosphate and ammonium biurate (Fig. 92). Vesical epithelium is common. Numerous bacteria are always present and they may be of many varieties: *Bacillus coli*, *Bacillus proteus vulgaris*, *Staphylococcus aureus* and *albus*, and others.

10. Vesical Calculus, Tumors, and Tuberculosis.—These conditions produce a chronic cystitis, with its characteristic urine. Blood, however, is more frequently present and more abundant than in ordinary cystitis. With neoplasms, especially, considerable hemorrhages are apt to occur. Particles of the tumor are sometimes passed with the urine. No diagnosis can be made from the

presence of isolated tumor cells. In tuberculosis tubercle bacilli can generally be detected.

11. Diabetes Insipidus.—Characteristic of this disease is the continued excretion of very large quantities of pale, watery urine, containing neither albumin nor sugar. The specific gravity varies between 1.001 and 1.005. The daily output of solids, especially urea, is increased.

12. Diabetes Mellitus.—The twenty-four-hour quantity of urine is very large as a rule, usually 4 or 5 liters in severe cases; and in general the volume varies directly with the percentage of sugar. Exceptionally it is above 25 liters. The color is generally pale, while the specific gravity in untreated advanced cases is

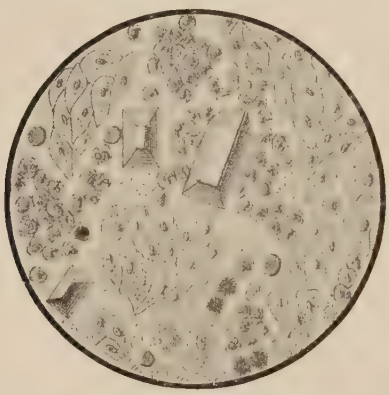


FIG. 92.—Sediment from cystitis (chronic): Numerous pus-corpuscles, epithelial cells from the bladder, and bacteria; a few red blood-corpuscles and triple phosphate and ammonium biurate crystals (Jakob).

nearly always high—1.030 to 1.050, very rarely below 1.020. Sometimes in mild or early cases the urine varies little from the normal in quantity, color, and specific gravity, and even very low specific gravity does not exclude diabetes.

The most characteristic sign of the disease is the presence of dextrose in the urine. In the vast majority of instances glycosuria means diabetes. In some extremely mild cases the amount is very small, and can sometimes be detected only in portions of urine secreted several hours after a meal rich in carbohydrates; in ordinary mild cases the urine regularly contains sugar, but can be rendered sugar free by the withdrawal of carbohydrates from the diet; in severe cases the glycosuria continues even when the carbo-

hydrates are completely withdrawn. The amount of dextrose in severe cases is usually 2 to 4 per cent. and may even exceed 8 per cent., while the total elimination may be 600 gm. in twenty-four hours. Acetone is generally present in moderately advanced cases, especially when the carbohydrate intake is restricted. Diacetic and oxybutyric acids may be present in the more severe cases and, if persistent, usually warrant an unfavorable prognosis. They are responsible for the acidosis which so frequently accompanies this disease and which may reach its culmination in diabetic coma. Accompanying the acidosis there is a corresponding increase in amount of ammonia; and, in general, estimations of ammonium salts will serve as an index of the degree of acidosis in diabetes.

13. Renal Glycosuria.—Recent studies indicate that this condition, sometimes wrongly called renal diabetes, is more common than has been recognized. The essential feature appears to be a lowering of the renal threshold for sugar.[1] The urine contains sugar, sometimes continuously, sometimes only after meals, while the blood-sugar remains within normal limits. Renal function is normal as regards the phenolsulphonephthalein test. The patient's health is affected little or not at all, and the condition may probably be regarded more as an anomaly than as a pathologic entity.

[1] Lewis, D. S., and Mosenthal, H. O.: Bull. Johns Hopkins Hosp., vol. 27, p. 133, May, 1916. Beard, A. H., and Grave, F.: Arch. Int. Med., vol. 21, p. 705, June, 1918. See also Schneiderman, H.: Jour. Amer. Med. Assoc., vol. 80, p. 825, March 24, 1923.

CHAPTER III

THE BLOOD

Preliminary Considerations.—The blood consists of a fluid of complicated and variable composition, the plasma, in which are suspended great numbers of microscopic structures, namely, red corpuscles, white corpuscles, blood-platelets, and blood-dust.

Red corpuscles, or **erythrocytes,** appear as biconcave disks, red when viewed by reflected light or in thick layer, and straw colored when viewed by transmitted light or in thin layer. They give the blood its red color. They are cells which have been highly differentiated for the purpose of carrying oxygen from the lungs to the tissues. This is accomplished by means of an iron-bearing protein, hemoglobin, which they contain. In the lungs hemoglobin forms a loose combination with oxygen, which it readily gives up when it reaches the tissues. Normal erythrocytes do not contain nuclei. They are formed from pre-existing nucleated cells in the bone-marrow. Their life, judged from the length of time that transfused corpuscles remain in the circulation in anemia, averages about eighty-three days (Wearn, Warren, and Ames). The total volume of the red corpuscles is slightly less than half that of the blood.

If a small drop of blood be taken upon a clean slide and covered with a clean cover-glass as in diagnosis of malaria (p. 314), the red corpuscles in the thicker portions of the preparation will often show a striking tendency to lie with overlapping edges, like piles of coins which have been tilted over (Fig. 93). Formerly much attention was paid to this *rouleaux formation* as a point in diagnosis of certain diseases, but it is now little regarded. Swift has recently found it absent in Hodgkin's disease, and, usually, in leukemia. Also, in such preparations of fresh blood, many of the red corpuscles are seen to be globular in shape and covered with knob- or spine-like processes (Fig. 94). This is called *crenation*, and has little or no clinical significance. It is favored by concentration of the fluid due to evaporation at the edge of the cover. Crenated corpuscles are often seen in concentrated urine and other body fluids and should always be recognized.

White corpuscles, or **leukocytes,** are less highly differentiated cells. There are several varieties. They all contain nuclei, and most of them contain granules which vary in size and staining properties. They are formed chiefly in the bone-marrow and lymphoid tissues. Their function is not fully understood. It ap-

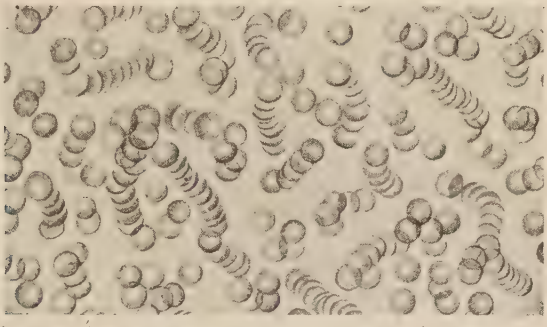


FIG. 93.—Unstained red blood-cells, showing rouleaux formation (Stengel and Fox; drawn under direction of C. Y. White).

pears to be concerned chiefly with the protection of the body against harmful agencies, in part through phagocytosis, in part through production of antitoxic substances and of ferments which play an important rôle in pathology.

Blood-platelets, or *blood-plaques*, are colorless or slightly bluish, spheric or ovoid bodies, usually about one-third or one-half

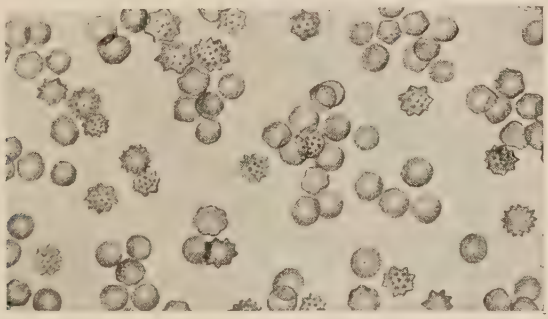


FIG. 94.—Unstained red blood-cells, showing crenation (Stengel and Fox; drawn under direction of C. Y. White).

the diameter of an erythrocyte, sometimes even as large as an erythrocyte. They appear to be constricted-off portions of the pseudopodia of certain giant-cells of the bone-marrow. Their function is not fully known, but is in some way connected with coagulation.

The **blood-dust of Müller** (*hemoconia*) consists of fine granules which have Brownian motion. The larger granules resemble micrococci. Little is known of them and they are given no consideration in clinical blood examinations. Most of them are probably minute fat globules, others granules from disintegrated leukocytes.

The **total amount** of blood, as shown by the method of Keith, Rowntree, and Geraghty,[1] averages about one-twelfth of the body weight. Roughly it may be put at 85 c.c. for each kilogram (2.2 lbs.) or about 5 or 6 liters for the average adult. Little attention is paid to this subject in clinical work, but it is clear that fluctuations in volume, which are common in pathologic conditions, must have a marked effect upon metabolism and will also alter the percentage of hemoglobin and the blood-cell count.

The **reaction** is faintly alkaline and is maintained throughout life at a remarkably constant level, as is explained in the section on Acidosis, page 563.

The **color** is due to the presence of hemoglobin in the red corpuscles, the difference between the bright red of arterial blood and the purplish red of venous blood depending upon the relative proportions of oxyhemoglobin and reduced hemoglobin. The depth of color depends upon the amount of hemoglobin. In very severe anemias the blood may be so pale as to be designated as "watery." The formation of carbon-monoxid-hemoglobin in coal-gas-poisoning gives the blood a bright cherry-red color, while formation of methemoglobin in poisoning with potassium chlorate and certain other substances gives a chocolate color.

The clear, pale, straw-colored fluid which remains after coagulation (p. 225) and separation of the clot is called **serum.** In the serum are found the numerous substances which the tissues elaborate for protection against bacterial and other harmful agents. In most cases these substances, or "antibodies," are elaborated only when the harmful agent is present in the body, and they are "specific," that is, they are effective only against the one disease which has called them forth. A test for the presence of the antibody is, therefore, a test for the existence of the particular disease. The various tests based upon these principles have within recent

[1] Keith, N. M., Rowntree, L. G., and Geraghty, J. T.: A Method for the Determination of Plasma and Blood Volume, Arch. Int. Med., vol. 16, p. 547, October, 1915.

years become a very important part of clinical laboratory work. They are discussed in the chapter upon Serodiagnostic Methods.

Hemoglobin estimations and cell counts can be made with fair accuracy upon blood obtained from a vein and immediately shaken with a trace (about 0.05 gm.) of powdered neutral potassium oxalate to prevent clotting. This makes possible the study of the blood of a single patient by an entire class. Differential leukocyte counts made in this way are less accurate. After twenty-four hours results are unsatisfactory.

Clinical study of the blood may be discussed under the following heads: I. Methods of obtaining blood for examination. II. Coagulation. III. Hemoglobin. IV. Enumeration of erythrocytes. V. Color index. VI. Volume index. VII. Enumeration of leukocytes. VIII. Enumeration of platelets. IX. Study of stained blood. X. Blood parasites. XI. Tests for recognition of blood. XII. Isohemagglutination groups. XIII. Blood chemistry. XIV. Miscellaneous methods. XV. Special blood pathology.

I. METHODS OF OBTAINING BLOOD

For most clinical examinations only one drop of blood is required. This may be obtained from the lobe of the ear, the palmar surface of the tip of the finger, or, in the case of infants, the plantar surface of the great toe or of the heel. In the case of the ear, the edge of the lobe, not the side, should be punctured. With bedridden patients the finger will be found most convenient, otherwise the ear is preferable, as it is less sensitive. An edematous or congested part should be avoided; also a cold, apparently bloodless one. The site should be well rubbed with alcohol to remove dirt and epithelial débris and to increase the amount of blood in the part. After allowing sufficient time for the circulation to equalize, the skin is punctured with a blood-lancet (of which there are several patterns upon the market) or some substitute, as a large Hagedorn needle, hypodermic needle, trocar, a spicule of glass, or a pen with one of its nibs broken off. The Hagedorn needle or the hypodermic needle may be recommended as being cheap, easily obtained, and fully as efficient as an expensive lancet. Either may be fixed in the cork of a small vial of alcohol and thus kept immersed in the fluid. Nothing is more unsatisfactory than an ordinary round sewing-needle. The lancet should be cleaned with

alcohol before and after using, but need not be sterilized. The puncture is practically painless if properly done with a sharp needle. It is made *with a firm, quick stab*, which, however, must not be so quick nor made from so great a distance that its site and depth are uncertain. The depth may be guarded with the thumb-nail if the lancet is not provided with a guard, but this should not be necessary. The first drop of blood (Fig. 95) which appears should be wiped away, and the second used for examination. The skin at the site of the puncture must be dry, else the blood will not form a rounded drop as it exudes. The blood should not be

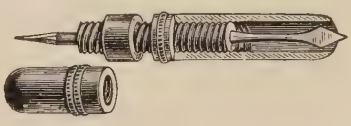


Fig. 95.—Daland's blood-lancet.

pressed out, since this dilutes it with serum from the tissues; but moderate pressure some distance above the puncture is allowable.

For serologic, bacteriologic, and chemical examinations a larger amount of blood is required. When 10 to 20 drops will suffice they can be obtained from a deep puncture of the lobe of the ear. For this, a spring-lancet (Fig. 96) is best. Larger amounts are usually drawn from a vein as described below. For some purposes, particularly in children when puncture of a vein is not practicable, the blood can be obtained by means of a "wet cup."

Fig. 96.—Spring-lancet.

Method of Obtaining Blood from a Vein.—Prepare the skin at the bend of the elbow as for a minor operation, or simply rub well with 70 per cent. alcohol or paint with tincture of iodin. The iodin is efficient as a germicide, but makes it more difficult to see the vein.

Bind a rubber or muslin bandage firmly around the upper arm, The end is tucked under the last round in such a manner that a slight pull will release the bandage. With a rubber bandage one turn will suffice (Fig. 97). The cuff of the blood-pressure apparatus answers admirably. Instead of a bandage it will often be sufficient for an assistant or even the patient to grasp the upper arm firmly.

Have the patient extend his arm fully and open and close the fist a few times to cause the veins to become distended. Even if not seen, they can usually be felt as cords beneath the skin. In fat persons veins

FIG. 97.—Showing method of obtaining blood from a vein.

which show as blue streaks are usually too superficial and too small. Grasp the forearm with the left hand, draw the skin tense with the

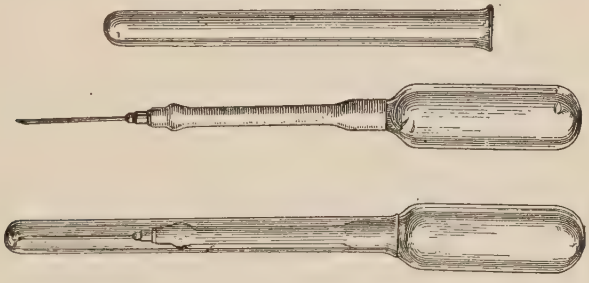


FIG. 98.—Keidel's vacuum tube for collecting blood from a vein, consisting of a sealed ampule, a needle with rubber connection, and a small glass test-tube to serve as cap. After the needle has entered the vein the stem of the ampule is crushed within the rubber connection and blood enters because of the vacuum. Similar tubes containing sterile culture-media are upon the market. (Courtesy of Hynson, Westcott, and Dunning, Baltimore, Md.)

thumb, and insert a sterile hypodermic needle attached to a sterile syringe into any vein that is prominent. The needle should be large—about 19 to 21 gage. It should go through the skin about 3 mm. from

the vein with the bevel at its tip uppermost and should enter the vein obliquely from the side, this requiring two movements, one to puncture the skin, one to enter the vein. If the needle is pushed through the skin directly over the vein, the vein is likely to roll to one side, thus escaping the needle.

When sufficient blood is obtained the bandage is first removed and the needle is then withdrawn, this order being followed to avoid formation of a hematoma. It is usually easy to secure 5 to 15 c.c. of blood. *If the needle be sharp and smooth* the procedure causes the patient surprisingly little inconvenience, seldom more than does an ordinary hypodermic injection. Dull needles may be sharpened by rubbing the bevel, point forward, on a hard, fine oil-stone, and then smoothed with Bon Ami. Platinum needles are greatly to be preferred to steel.

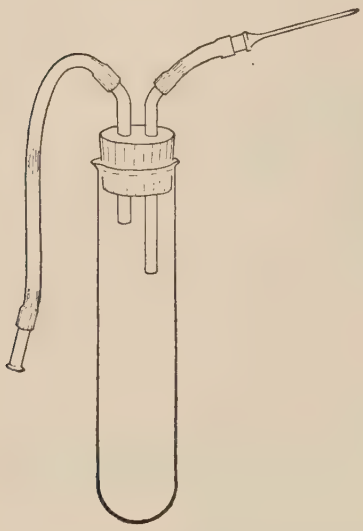


FIG. 99.—Device for drawing blood from a vein, using a large test-tube, a centrifuge tube, or a small flask. It may be modified in various ways. The needle may be attached directly to the glass tube which is ground to fit it (Cummer), thus doing away with the rubber connection; or the hub of the needle may be fixed directly in the rubber stopper in place of the glass tube (Woolley).

There is rarely any difficulty in entering a vein except in children and in adults when the arm is fat and the veins are small. If desired, one of the veins about the ankle can be used. In the case of infants blood may be secured from the superior longitudinal sinus by puncturing through the posterior angle of the anterior fontanel. A short needle (about $\frac{3}{8}$ inch) of rather large caliber should be used.

Instead of a syringe many other devices for securing blood from a vein may be employed. Two of these are shown in Figures 98 and 99, which indicate their construction in sufficient detail. They possess the advantage that the blood can be drawn directly into any desired reagent or culture-medium.

II. COAGULATION

Coagulation consists essentially in the transformation of fibrinogen, one of the proteins of the blood-plasma, into fibrin by means of a ferment called thrombin. The presence of calcium salts is necessary. The resulting coagulum is made up of a meshwork of

fibrin fibrils with entangled corpuscles and platelets. The clear, straw-colored fluid which is left after separation of the coagulum is called *blood-serum*.

Because of its great interest in connection with the pathogenesis and differential diagnosis of the primary and secondary hemorrhagic diseases the mechanism of coagulation must be considered more in detail. The process is complicated and not fully understood, and the theories which have been offered to explain it are many and intricate. It will suffice to outline Howell's theory, which is one of the simplest and serves well as a basis for clinical work. Five "coagulation factors" are assumed to take part. Four of these—fibrinogen, calcium salts, prothrombin, and antithrombin—are normal and constant constituents of the blood-plasma. Prothrombin is held in some sort of combination with antithrombin and is by this means kept inactive so that coagulation within the vessels cannot occur. The fifth coagulation factor, thromboplastin, a coagulation-accelerating substance, is not present in the circulating blood, but resides in the tissue juice outside the blood-vessels. When the blood escapes from the vessels it meets the thromboplastin of the tissues, and this at once neutralizes the antithrombin and thereby sets free prothrombin. The prothrombin thus released is then activated by calcium salts and becomes thrombin, a ferment-like substance whose function it is to transform fibrinogen of the plasma to fibrin. The fibrin forms a meshwork of threads in which the cellular elements are entangled, and thus constitutes the essential part of the coagulum. After a time the coagulum contracts, pressing out the serum.

Thus the process of coagulation takes place in three stages: (*a*) Neutralization of antithrombin and the formation of thrombin from the released prothrombin, these processes being invisible; (*b*) transformation of fibrinogen into fibrin, which is the visible evidence of clotting; (*c*) retraction of the clot. All the substances concerned in clotting except one are normally present in the plasma; that one, thromboplastin, resides outside the vessels in tissue juice. To explain coagulation of blood within the vessels, or of blood removed directly from a large vein without possible addition of tissue juice, it is assumed that enough thromboplastin to neutralize antithrombin and thus initiate clotting may be formed from disintegration of blood-platelets. The much more rapid clotting of blood from an ordinary puncture is explained by the greater contamination with tissue juice.

Red cells and leukocytes take little or no active part in coagulation, but are passively entangled in the coagulum. Blood-platelets, upon the

other hand, are intimately connected with the process, and this seems to be their chief function (p. 264).

For certain purposes, notably for bacteriologic and chemical work, it is necessary to prevent coagulation of the blood which is withdrawn. This may be accomplished by receiving it directly in a solution of 1 per cent. sodium citrate (or ammonium oxalate) in physiologic salt solution, or into a tube containing a very little finely powdered neutral potassium oxalate. This precipitates calcium salts or renders them unavailable for purposes of clotting.

In clinical work five features of the clotting process are studied in appropriate cases: coagulation time, character of the clot, prothrombin time, calcium time, and bleeding time. These are discussed in the following paragraphs:

1. Coagulation Time.—Normally, when blood is secured from an ordinary skin puncture, coagulation takes place in two to six minutes after it leaves the vessels, usually about four and a half minutes. The time is influenced by temperature, size of the drop, smoothness and cleanliness of the instruments, and other factors. Clotting is more rapid after meals. It is much more rapid when the blood is squeezed from a puncture than when it flows freely, owing to admixture with tissue juice. It is never possible to estimate the amount of such admixture when a skin puncture is the source of the blood. *For this reason, if results are to be relied upon, it is imperative that the blood be taken from a vein.* The normal coagulation time is then considerably longer, averaging about twenty minutes by Howell's method, five to ten minutes by Lee and White's; while in some pathologic conditions the discrepancy may be much greater. Lee and White cite an instance in which hemophilic blood with a true coagulation time of fifty minutes coagulated in five minutes when secured from an ear prick.

Shortening of coagulation time, which in some cases may be due to deficiency of antithrombin, is not of much clinical significance except in relation to possible thrombosis, as in typhoid. *Prolongation* is important. The greatest prolongation occurs in hemophilia, where it is usually one to several hours. There is less, though still marked, delay in melena neonatorum; in obstructive jaundice; and, less important clinically, in some anemias and leukemias, and in many infectious diseases, notably pneumonia. Many apparently healthy individuals have coagulation time some-

what beyond the usually accepted normal limit. Aside from its value in the diagnosis of hemorrhagic diseases, estimation of coagulation time is important as a preliminary to operation when there is any reason to expect dangerous capillary oozing, as in tonsillectomies or operations upon jaundiced persons. It should always be supplemented by estimation of the bleeding time (p. 231).

There are many methods of ascertaining the coagulation time, and results by the different methods are not comparable because of difference in their end-points and in the conditions to which the blood is subjected. It is therefore well to adopt a single method for one's routine work and to keep all conditions as nearly uniform as possible. In every case absolute cleanliness of the instruments is imperative. It is always advisable to test the blood of a normal person under exactly the same conditions as a control.

Methods Which Use Blood from a Skin Puncture.—The puncture should be deep enough to ensure free flow of blood in order to lessen contamination with tissue juice. The first drop should be wiped off and the second used for the test. Time is counted from the first appearance of the drop.

The simplest method is to receive several drops of blood (well rounded drops 4 to 5 mm. in diameter) on a clean slide and to draw a needle through one or another of the drops at one-minute intervals. When shreds of fibrin cling to the needle and are dragged along by it coagulation has taken place. The slide may be kept inverted over a glass of warm water in order to avoid evaporation, which in a dry climate may entirely vitiate results. Duke uses a glass slide to which two glass disks 5 mm. in diameter are cemented. Well-rounded drops of blood—one from the patient, one from a normal person—are received on the disks, and the slide is inverted across the top of a glass or beaker containing water at 40° C. and covered with a towel. Coagulation is judged by the shape of the drops when the slide is held in a vertical position (Fig. 100).

A satisfactory method is to take up the blood in a capillary glass tube about 1.5 mm. in diameter. If clean, the tube fills readily by capillary attraction. Short sections of the tube are then carefully broken off at one-minute intervals after scratching with a file, and the ends are gently separated. When coagulation has occurred threads of fibrin will be seen to span the gap between the broken ends. Some definite end-point should be adopted, as for example, when the fibrin will span a gap of 5 mm.

There are many methods which require special instruments, of which the best known are those of Biffi-Brooks and of Boggs (Fig. 101). Directions for use of these can be obtained from the makers. They offer no great advantages over the methods given above.

Methods Which Use Blood from a Vein.—The time is counted from the first appearance of the blood in the syringe. Any of the methods mentioned above may be used, the drops of blood being transferred from the syringe to the appropriate instrument, but the following are more satisfactory:

Lee and White's Method.—Secure the blood with a small hypodermic syringe, entering the vein as quickly as possible, and avoiding

FIG. 100.—Showing difference in shape of blood-drops before coagulation (upper drop), and after coagulation (lower drop) (Duke's method).

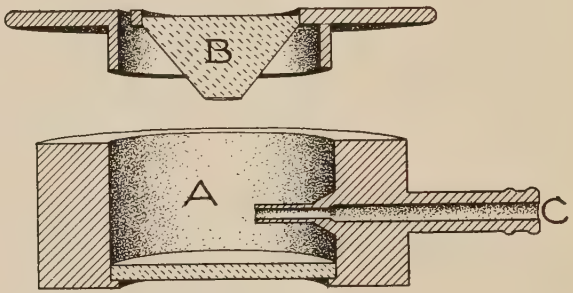


FIG. 101.—Boggs' coagulation instrument: *A*, Metal box with glass bottom; *B*, glass cone; *C*, tube through which air is blown by means of a rubber bulb not shown in the figure. A rounded drop of blood is placed upon the small end of the cone and this is quickly inverted into the box. The instrument is placed upon the microscope and the edge of the blood-drop nearest the air-tube is viewed with the 16-mm. objective. Gentle puffs of air are then blown against the blood-drop at half-minute intervals. Coagulation is assumed to be complete as soon as the corpuscles move *en masse* in a radial direction and spring back to their original position.

suction. Place 1 c.c. of blood in a test-tube 8 mm. in diameter, which has been rinsed with physiologic salt solution just previously. Place the tube in a glass of water at about 75° F., although this is unnecessary if the room temperature lies between 65° and 90° F. Tilt the tube at intervals. Coagulation is assumed to be complete as soon as the tube can be inverted without displacing the clot. The normal coagulation time by this method is five to ten minutes. The greater the diameter of the tube, the slower the clotting.

Howell's method is similar. Fill a hypodermic syringe and its attached needle with a mixture of ether and petrolatum, force it out,

and draw air into the syringe a few times. The ether will evaporate, leaving a thin coating of petrolatum. Secure 2 to 4 c.c. of blood with this syringe and place it in a test-tube about 21 mm. in diameter. The end-point is judged by tilting and finally inverting the tube as in Lee and White's method. By this method the blood of healthy persons was found to coagulate in ten to thirty minutes, average about twenty minutes.

The method of *King and Murray* uses a device resembling a 5-c.c. glass syringe-barrel with no plunger and with a metal stop-cock inter-

FIG. 102.—King-Murray coagulation tube.

FIG. 103.—Normal clot retraction.

posed between the barrel and the needle (Fig. 102). The needle is inserted into the arm vein, and when the blood has risen to the 2 c.c. mark the stop-cock is closed and the needle withdrawn. The instrument is then tilted at intervals and finally inverted as in the methods just described. The normal coagulation time by this method is usually nine to eleven minutes, with eight and fifteen minutes as normal extremes.

2. Character of the Clot.—Under normal conditions the coagulum begins to retract within a few hours after it is formed,

gradually separating from the wall of the vessel in which it is contained and expressing serum (Fig. 103). The process is completed within eighteen to twenty-four hours.

For the study of retraction 2 or 3 c.c. of blood are taken in a test-tube, placed in an incubator at 37° C., and observed at intervals for a day or two. The specimen of blood used in the coagulation tests just described may be further utilized for this purpose.

The phenomenon of retractility is apparently due to presence of blood-platelets, for the degree of retractility closely parallels the number of platelets (p. 264). It is entirely independent of the coagulation time. This is most typically exemplified in purpura hæmorrhagica (p. 389). The coagulum is formed within the normal time, but it retracts very little or not at all even after standing several days. In hemophilia, upon the other hand, with normal number of platelets, the blood coagulates very slowly, but the clot when once formed has normal retractile power.

3. Prothrombin Time.—For the differentiation of the various conditions in which the clotting mechanism is disturbed it is necessary to study not only the coagulation time but also to determine quantitatively the various coagulation factors: prothrombin, antithrombin, fibrinogen, calcium, and so forth. Of these, prothrombin has had great clinical interest. A fair idea of the relative amount or strength of prothrombin was thought to be given by the simple method of Howell detailed below.

The normal "prothrombin time" by this method averages about ten minutes. It is constantly and very markedly prolonged in hemophilia—a fact which, according to Hurwitz and Lucas, makes the diagnosis of this disease relatively simple. The usual "prothrombin time" of hemophilic blood is five to twenty-five times the normal.

Recently, however, Howell and Cekada[1] showed that prothrombin is normal in quantity and in properties in hemophilia. The platelets are resistant, and because they do not disintegrate there is a lack of thromboplastic material. It is apparent then that the "prothrombin time" test does not indicate relative amounts of prothrombin; nevertheless, the procedure is still of value in the diagnosis of hemophilia.

[1] Howell, W. H., and Cekada, E. B.: The Cause of the Delayed Clotting of Hemophilic Blood, Amer. Jour. of Physiology, vol. 78, p. 500, November, 1926.

Howell's Method for "Prothrombin Time."—1. Obtain about 2 c.c. of blood from a vein, using a syringe which has been rinsed out with physiologic salt solution. Avoid suction.

2. At once place the blood in a centrifuge tube which contains 0.25 c.c. of 1 per cent. sodium oxalate in physiologic salt solution.

3. Mix by inverting several times and centrifugalize thoroughly.

4. Place 5 drops of the clear plasma in each of four small test-tubes.

5. To these tubes add 0.5 per cent. solution of calcium chlorid in increasing quantities: 2 drops in Tube 1; 3 drops in Tube 2; 4 drops in Tube 3; 5 drops in Tube 4. Mix gently.

6. Coagulation will probably occur in all tubes, but not at the same rate. Its occurrence is recognized by invertibility of the tube as in Howell's coagulation method. The coagulation time of the tube which clots earliest is the "prothrombin time." With each unknown blood a normal is run. The "prothrombin quotient" of Hurwitz and Lucas is found by dividing the time of the unknown by that of the normal control.

4. Calcium Time.—When the coagulation time is abnormally slow, especially in jaundiced patients, it may be desirable to find whether the delay is due to deficiency of calcium and thus whether clotting can probably be hastened by administration of calcium.

To this end, a few cubic centimeters of blood are obtained from a vein, and 1 c.c. is placed in each of two test-tubes 8 to 10 mm. in diameter. To one of these is added 3 drops of 1 per cent. solution of calcium chlorid (or 6 drops of 0.5 per cent. solution). Should the blood in the tube containing the calcium coagulate within the normal time, while coagulation in the tube without calcium is delayed, the delay may be assumed to be due, in part at least, to deficiency of calcium in the blood.

5. Bleeding Time.—This is a term used by Duke to indicate the time required for a small cut to cease bleeding. It does not necessarily parallel the coagulation time of the blood; for it is largely dependent upon the efficiency of the tissue juice in accelerating clotting, upon the elasticity of the skin, and upon the mechanical and chemical action of the blood-platelets. The normal bleeding time is one to three minutes, although it may sometimes be as long as eight minutes. There is slight delay—usually five to ten minutes—in many severe anemias. Duke found great prolongation, ten to ninety minutes or longer, in two classes of conditions: (*a*) those in which the blood-platelets are very greatly reduced,

notably purpura hæmorrhagica; and (*b*) those in which the fibrinogen content of the blood is extremely low, as chloroform- and phosphorous-poisoning and certain destructive diseases of the liver with a hemorrhagic tendency. Contrary to common belief the bleeding time by this method is usually not prolonged in hemophilia (p. 389).

In surgery the bleeding time may well be studied in connection with the coagulation time as a routine procedure before operation, but it should not take its place.

Duke's Method for Bleeding Time.—1. Make a slight cut in the lobe of the ear. The usual puncture as made for a blood-count will answer if fairly deep, although it is generally best to use the point of a scalpel. Within wide limits the size of the cut is not important. It is most satisfactory when it gives a blot about 1 or 2 cm. in diameter at the end of the first half-minute.

FIG. 104.—A strip of soft filter-paper showing a row of blood-blots obtained in a test of the bleeding time by Duke's method. Blots are made at half-minute intervals. In this case the bleeding time is normal (photograph natural size).

2. At half-minute intervals blot with a piece of absorbent paper all the blood which has flowed out. This furnishes a series of blots and the rate of decrease in size indicates the rate of decrease of the hemorrhage. The usual bleeding time is one to three minutes, giving two to six blots (Fig. 104). In certain pathologic conditions mentioned above bleeding may continue for an hour or more. When the time is moderately prolonged the twentieth blot will be about one-half the size of the first; when it is enormously prolonged the twentieth blot may be fully as large as the first.

III. HEMOGLOBIN

Hemoglobin is an iron-bearing protein which normally occurs in the circulating blood in two forms: *oxyhemoglobin*, chiefly in arterial blood; and *reduced* hemoglobin (more correctly called simply *hemoglobin*), chiefly in venous blood. Through the action of acids,

alkalies, oxidizing and reducing substances, heat, and other agencies it is readily converted into a series of derivative compounds which can be distinguished by means of the spectroscope.

Most of these derivative compounds are formed only in blood which has left the vessels; a few, however, may be produced in the circulation.

Methemoglobin is formed in the circulating blood in most cases of the rare condition known as "enterogenous cyanosis," sulphhemoglobin having been found in a few other cases. Methhemoglobin is also found in poisoning with potassium chlorate, nitrites, nitrobenzol (as in shoe dye), acetanilid, phenacetin, and other substances. Clinically there is marked cyanosis, and in severe cases the blood has a chocolate-brown color when withdrawn. When the condition is at all marked methemoglobinemia is easily recognized spectroscopically (p. 325).

Carbon monoxid hemoglobin, formed in carbon monoxid poisoning, gives the blood a brighter red color than is normal.

Acute poisoning has long been well known. Chronic poisoning, due to prolonged exposure to small amounts of carbon monoxid, is less well known, but is assuming increasing importance. The chief sources of the gas are gasolene motors, illuminating gas, gas heaters, and defective stoves and furnaces. Exposure to carbon monoxid is thus one of the hazards of modern civilization. It has even been found in the air of busy streets of large cities in sufficient concentration to cause mild symptoms in persons, such as traffic policemen, who are long exposed to it.

Henderson and Haggard found that healthy persons exposed to various concentrations of the gas for an hour did not experience definite symptoms (headache, dizziness, muscular weakness, nausea) unless the concentration in the blood reaches 26 or 30 per cent. of saturation, but it appears that in chronic poisoning, especially in children, serious symptoms may occur with less amounts. The figures reported for clinical cases of poisoning are often misleading, since the carbon monoxid largely or wholly disappears from the blood after the patient has breathed pure air for a few hours, although the symptoms may continue for a long time. CO-hemoglobin has a characteristic spectrum (p. 324), and, when present in sufficient amount (30 per cent. or more), is readily identified with the ordinary "pocket" spectroscope, but chemical tests are much more sensitive and more satisfactory. Two simple qualitative tests are described below. For a relatively simple quantitative method the reader is referred to Sayers and Yant.[1]

[1] Sayers, R. R., and Yant W. P.: The Tannic Acid Method for Quantitative. Determination of Carbon Monoxid in the Blood, U. S. Public Health Reports, Treasury Department, vol. 37, No. 40, p. 2433, October 6, 1922. See also Jour. Amer. Med. Assoc., vol. 78, p. 1745, June 3, 1922.

Katayama's test is one of the best. It will detect as little as 10 per cent. of saturation. Place about 10 c.c. of water in each of two test-tubes. To one add 5 drops of the suspected blood, and to the other 5 drops of normal blood to serve as a control. To each tube add 5 drops of fresh orange-colored ammonium sulphid, mix gently, and make faintly acid with acetic acid. Blood containing carbon monoxid hemoglobin develops more or less rose-red color, depending on the concentration; normal blood, a dirty greenish brown.

Hoppe-Seyler's test is less sensitive and less satisfactory, but, since it requires no chemical excepting sodium hydroxid, is useful in emergencies. It may be performed as follows: To 3 c.c. of water in a test-tube add 3 to 5 drops of the blood and 1 drop of 5 per cent. sodium hydroxid solution, mix gently, and let stand one hour. Normal blood gives a greenish-brown color, carbon monoxid blood more or less pink. It is always necessary to run a control with normal blood.

Normally hemoglobin is confined to the red corpuscles. When it is dissolved out of these cells and appears in the plasma the condition is known as *hemoglobinemia*. This occurs in a great variety of conditions, among which may be mentioned: severe types of infectious diseases; paroxysmal hemoglobinuria; severe burns and frost bites; and poisoning with potassium chlorate, mushrooms, and some other poisons. When the free hemoglobin reaches a certain concentration in the blood-plasma it is excreted in the urine (hemoglobinuria).

To recognize hemoglobinemia, receive a little blood in a small dry test-tube and allow it to stand in a cool place for twenty-four hours. The serum, which separates after coagulation, will be colored red or pink instead of pale yellow, as is normally the case.

The normal amount of hemoglobin was formerly given as about 14 gm. for each 100 c.c. of blood, but recent work has shown this to be too low for adults. The general average for both sexes during adult life appears to be about 16 gm. The absolute amount is, however, seldom estimated clinically: it is the relation which the amount present bears to an arbitrarily fixed normal that is determined. Thus the expression, "50 per cent. hemoglobin," when used clinically, means that the blood contains 50 per cent. of this normal. Practically, however, with the various methods of estimation in general use the blood of healthy adults ranges from 80 to 105 per

cent.; these figures may, therefore, be taken as representing normal limits. There are, moreover, marked fluctuations with age and sex, which must be taken into account in any careful case study. These are well shown in Figure 105, which is based upon Williamson's careful spectrophotometric study of the blood of 919 healthy persons in Chicago.

The custom of recording hemoglobin in terms of percentage of an indefinite normal is grossly inaccurate and leads to much confusion.

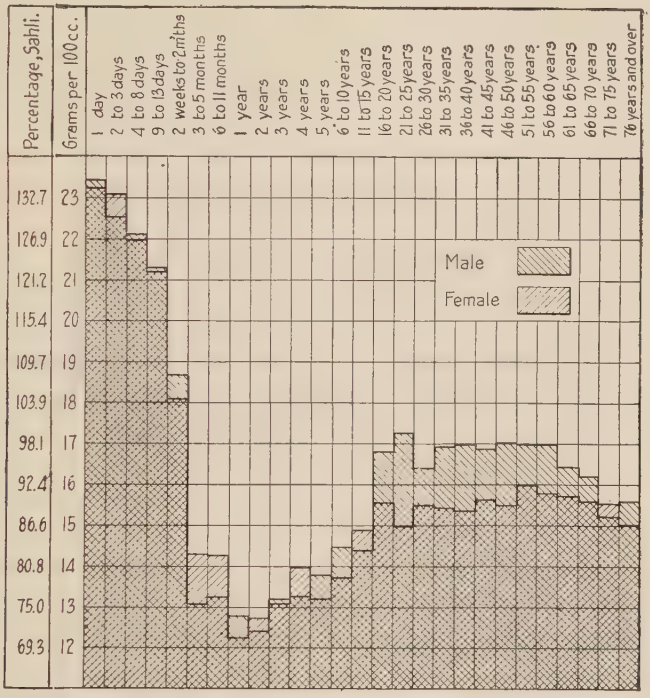


FIG. 105.—Diagram showing average hemoglobin values for both sexes at different ages (after Williamson). Some have obtained slightly lower values for the different ages. The corresponding percentages on the Sahli hemoglobinometer, which assumes 17.3 gm. for each 100 c.c. blood to be the normal, are also shown.

From what has just been said it is clear that no single normal standard can be applied to all ages and both sexes. The situation is complicated by the fact that different hemoglobinometers use different standards. A blood, for example, which reads 100 per cent. on the Dare should read about 80 per cent. on the Sahli if both instruments be calibrated according to the makers' claims. A record, therefore, means little unless one knows what instrument was used and the age and sex of the patient.

This confusion could be avoided if records were made in terms of the actual percentage of hemoglobin, that is, in grams for each 100 c.c. of blood. This plan has been adopted by certain large hospitals, and has been proposed to the American Society of Clinical Pathologists for consideration as a standard method. The reading on any type of instrument can readily be converted into absolute percentage if one knows what amount of hemoglobin was adopted by the makers as normal, provided, of course, that the particular instrument is accurately standardized. This calculation will be given with the description of the various instruments.

Increase of hemoglobin, or *hyperchromemia*, is uncommon, and is usually more apparent than real. It accompanies an increase in number of erythrocytes, and may be noted in change of residence from a lower to a higher altitude; in poorly compensated heart disease with cyanosis; in concentration of the blood from any cause, as the severe diarrhea of cholera; and in "idiopathic polycythemia" or erythremia.

Decrease of hemoglobin, or *oligochromemia*, is very common and important. It is the distinctive and most striking feature of the anemias (p. 368). In secondary anemia the hemoglobin loss may be slight or very great. In mild cases a slight decrease of hemoglobin is the only blood change noted. In very severe cases, especially in repeated hemorrhages, malignant disease, and infestation by the hookworm and *Diphyllobothrium latum*, hemoglobin may fall to 15 per cent. Hemoglobin is always diminished, and usually very greatly, in chlorosis (average about 40 to 45 per cent.), pernicious anemia (average about 20 to 25 per cent.), and leukemia (usually about 40 to 50 per cent.).

Estimation of hemoglobin is less tedious and, if reasonably accurate, usually more helpful than a red corpuscle count. It offers the simplest and most certain means of detecting the existence and degree of anemia, and of judging the effect of treatment in anemic conditions. Pallor observed clinically does not always denote anemia.

There are many methods, but none is entirely satisfactory. With the different standards for the normal adopted by different makers and the inaccuracies and deterioration of individual instruments records of hemoglobin estimations are generally untrustworthy. *The physician should standardize his instrument to give readings of 100 per cent. upon the bloods of healthy adults whose red*

corpuscles number 5,000,000 for each cubic millimeter. If the average reading falls above or below 100, a correction-factor must be worked out. Such standardization must be repeated several times a year. The methods of estimating hemoglobin which are most widely used are here described:

1. **Tallqvist Method.**—The popular Tallqvist hemoglobinometer consists simply of a book of small sheets of absorbent paper and a carefully printed scale of colors (Fig. 106).

Take up a large drop of blood with the absorbent paper, and when the humid gloss is leaving, before the air has darkened the hemoglobin, compare the stain with the color scale. One should stand with the back to the window and with the light falling over one shoulder. The color which it matches gives the percentage of hemoglobin. In practised hands and with a well-printed scale this method is accurate to within about 10 per cent. The Tallqvist scale is so inexpensive and so convenient that it should be used by every physician at the bedside and in hurried office work; but it should not take the place of the more accurate instruments.

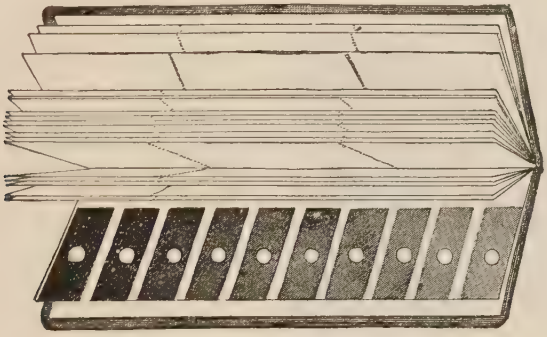


FIG. 106.—Tallqvist's hemoglobin scale.

2. The **Sahli hemoglobinometer** (Fig. 107) consists of an hermetically sealed comparison tube containing a suspension of acid hematin, a graduated test-tube of the same diameter, and a pipet of 20-cu. mm. capacity. The two tubes are held in a black frame with a white ground-glass back.

Place decinormal hydrochloric acid solution in the graduated tube to the mark 10. Obtain a drop of blood and draw it into the pipet to the 20-cu. mm. mark. Wipe off the tip of the pipet, blow its contents into the hydrochloric acid solution in the tube, and rinse well. The

hemoglobin is changed to acid hematin. Place the two tubes in the compartments of the frame; let stand one minute, and dilute the fluid with water drop by drop, mixing after each addition, until it has exactly the same color as the comparison tube. The graduation corresponding to the surface of the fluid then indicates the percentage of hemoglobin. Mixing may be done by closing the tube with the finger and inverting, but care should be exercised to see that none of the fluid is removed by adhering to the finger. Slightly waxing the finger will aid. Decinormal hydrochloric acid solution is prepared with sufficient accuracy for this purpose by adding 1 c.c. of the concentrated acid to 99 c.c. distilled water. A little chloroform should be added as a preservative.

Under favorable conditions this method is very satisfactory, and is probably accurate to within 5 per cent. Unfortunately, not all instruments upon the market are well standardized, and the comparison tube does not keep its color well. Sometimes, however, the apparent fading is due to the fact that the hematin is in suspension and settles out when the instrument lies unused for some time. This can be remedied by inverting the tube a number of times. Most tubes contain a glass bead to facilitate mixing. The color-tube should be kept from the light as much as possible.

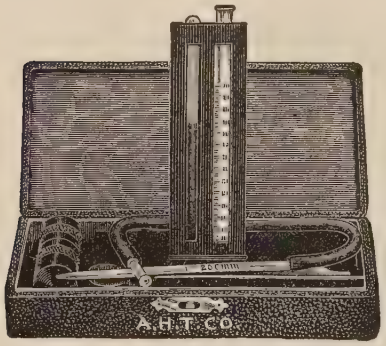


Fig. 107.—Sahli's hemoglobinometer.

A reading of 100 per cent. on the Sahli instrument should, according to the makers, correspond to 17.3 gm. of hemoglobin in 100 c.c. of blood. The blood of normal adults therefore reads 80 to 90 per cent.

The instrument should be tested every six months with the bloods of a series of normal persons whose red corpuscles average 5,000,000 for each cubic millimeter. When the standard has faded perceptibly it may be renewed by breaking off the tip of the tube (after nicking with a file) and replacing the fluid with the following:

Blood of a healthy individual having red cell count of 5,000,000	0.1 c.c.
Decinormal hydrochloric acid	1.0 "
Water, to	5.0 "
Glycerol	5.0 "

The tube is then stoppered with a paraffined cork. If a new tube be used, it is very important that the standard and graduated tubes have

the same inside diameter. The new Sahli-Leitz modification of this instrument discards the tube of fluid and substitutes a rod of colored glass which is claimed to be non-fading. The value of the scale in grams of hemoglobin for each 100 c.c. of blood is not given by the makers, which is a decided disadvantage.

3. **Dare's hemoglobinometer,** which has been widely used for many years, differs from the others in using undiluted blood. Its general appearance and the different parts are well shown in Figure 108. The blood is allowed to flow by capillarity into a slit between two small plates of glass clamped in a small metal holder. One of the pieces is of milk-glass, the other clear. This "pipet" is then placed in the instrument, *with the milk-glass toward the source of light,* and compared, by looking through a tube, with different portions of a circular glass disk of graduated thickness which is revolved by means of a small wheel seen at the top of the illustration. The two colors appear side by side, and will show their true values only when viewed in a darkened room by the light of a candle or a small electric light. Usually a dark corner of the office will suffice. The reading is taken at the beveled edge of a slot shown in the figure, and it must be made quickly, before clotting takes place. One hundred per cent. upon the scale is said by the makers to correspond to 13.77 gm. of hemoglobin in each 100 c.c. of blood. In our experience the instrument tends to read a little low in the upper part of the scale.

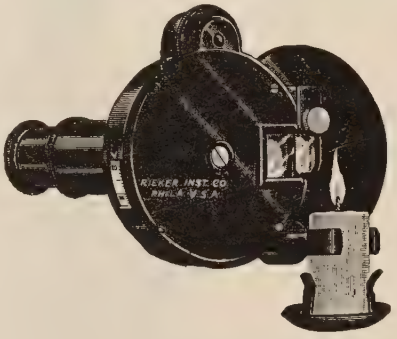


FIG. 108.—Dare's hemoglobinometer, illumination by candle. This model is much less satisfactory than the newer electric models, but is pictured here because it shows the construction more clearly.

Dare's instrument is easy to use and to clean; and, although most persons find it difficult to match shades of red accurately, it is, upon the whole, a satisfactory type of instrument for general clinical work. The colored glass shows little or no tendency to fade, and the instrument rarely needs restandardization. The electric model with small lamp and dry battery is especially convenient and satisfactory. Electric attachments can be purchased separately for use with the older models.

4. **Newcomer's Standard Disk.**—This consists of a small piece of light brown glass approximately 1 mm. thick, which may be used as a color standard for hemoglobin estimations with any colorimeter of the Duboscq type.

Place the disk in one of the light paths of the colorimeter, either

above one of the plungers or above or below one of the cups, and fill this cup with water.

In the other cup place exactly 5 c.c. of approximately decinormal hydrochloric acid (1 c.c. concentrated acid, 99 c.c. water). Secure exactly 20 cu. mm. of blood from a skin puncture (the pipet of the Sahli hemoglobinometer may be used), place the blood at once in the cup with the acid, and mix well. This gives a dilution of 1 in 251. Now adjust the plunger until the two fields match and make the reading. Calculate the amount of hemoglobin in grams for each 100 c.c. of blood by aid of the table on page 241, which takes into account two variable

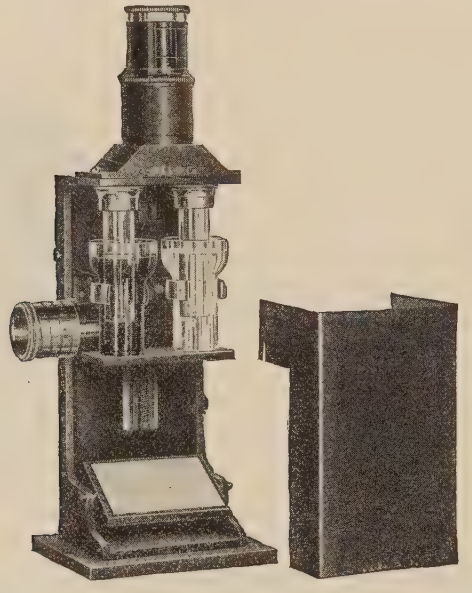


Fig. 109.—Newcomer's hemoglobinometer, a new and accurate instrument which utilizes Newcomer's disk. One hundred on the scale is equivalent to 16.92 grams of hemoglobin in each 100 c.c. of blood. (Bausch and Lomb Optical Co.)

factors: (*a*) the time which has elapsed since the blood dilution was made, the acid hematin solution reaching its full depth of color only after some hours; and (*b*), the thickness of the colored glass disk, which varies from 0.95 to 1.05 mm., the exact thickness being engraved upon each.

To translate grams hemoglobin for each 100 c.c. of blood into terms of percentage of normal, as is usually required in clinical work, multiply by 100 and divide by the number of grams hemoglobin which have been adopted as representing the normal (p. 235). For example, to express the hemoglobin in percentage according to Sahli's scale, multiply by 100 and divide by 17.3.

NEWCOMER'S TABLE FOR USE WITH THE STANDARD DISK

To Obtain Grams Hemoglobin Per 100 c.c. Blood Divide the Colorimetric Reading into the Appropriate Figure.

Minutes since dilution.	Thickness of the colored glass in millimeters.										
	0.95.	0.96.	0.97.	0.98.	0.99.	1.00.	1.01.	1.02.	1.03.	1.04.	1.05.
10	94.4	95.4	96.4	97.4	98.4	99.4	100.4	101.4	102.4	103.4	104.4
15	93.1	94.1	95.0	96.0	97.0	98.0	99.0	100.0	101.0	102.0	103.0
20	92.5	93.5	94.5	95.4	96.4	97.4	98.4	99.4	100.4	101.3	102.3
30	91.8	92.8	93.8	94.8	95.7	96.7	97.7	98.6	99.6	100.5	101.5
40	91.6	92 5	93.5	94.5	95.4	96.4	97.4	98.3	99.3	100.3	101.2
Final	90.6	91.6	92.5	93.5	94.4	95.4	96.4	97.3	98.3	99.2	100.2

With the carefully standardized disks at present supplied this method is perhaps the most accurate available for clinical purposes. Its use is, however, confined to laboratories equipped with an expensive colorimeter.

Recently, the Bausch and Lomb Optical Co. has introduced a new hemoglobinometer which utilizes Newcomer's method (Fig. 109).

5. **Van Slyke's Oxygen-capacity Method.**—This is an indirect method, which estimates the amount of hemoglobin from the amount of oxygen it will absorb and utilizes the Van Slyke apparatus described for CO_2 estimations (Fig. 164). It serves as an accurate standard method for standardizing the various hemoglobinometers, but is too complicated for clinical work. The technic will be found in Van Slyke's papers.[1]

IV. ENUMERATION OF ERYTHROCYTES

In health there are about 5,000,000 red corpuscles per cubic millimeter of blood. The number is generally a little less—about 4,500,000—in women. At birth the number averages about 7,000,000 and falls gradually to reach the adult figure at about the fifteenth year. This should be compared with the hemoglobin curve shown in Figure 105. Hawk finds the normal for athletes in training to be 5,500,000.

Increase of red corpuscles, or *polycythemia*, is unimportant. There is a decided increase following change of residence from a lower to a higher altitude, reaching a maximum after several days sojourn. The increase, however, is not permanent. In a few months the erythrocytes return to nearly their original number. At the University of Colorado (altitude 5400 feet) the average for healthy medical students is about 5,800,000. Several views have been offered in explanation of this effect of altitude: (*a*) concen-

[1] Van Slyke, D. D.: Jour. Biol. Chem., vol. 33, p. 127, January, 1918. Van Slyke, D. D., and Stadie, W. C.: Jour. Biol. Chem., vol. 49, p. 1, November, 1921.

tration of the blood, owing to increased evaporation from the skin; (*b*) altered distribution of corpuscles, the reserve cells in the splanchnic vessels being thrown into the peripheral circulation; (*c*) accumulation of red cells in the capillaries of the skin; (*d*) new formation of corpuscles or delayed destruction, this giving a compensatory increase of aëration surface. The work of Schneider at Colorado Springs and of Liebesny of Vienna with the skin microscope support the second and third of these views, although the fourth must be accepted to explain the moderate permanent increase.

Pathologically, polycythemia is uncommon. It may occur in: (*a*) concentration of the blood from severe watery diarrhea; (*b*) chronic heart disease, especially the congenital variety, with poor compensation and cyanosis; (*c*) acute poisoning by phosphorous, cantharides, or carbon monoxid; and (*d*) polycythemia rubra or erythremia (p. 383), which is considered to be an independent disease, and is characterized by a dark red cast of countenance, blood-counts of 7,000,000 to 12,000,000, hemoglobin 120 to 150 per cent., and a normal number of leukocytes. The highest count recorded is 15,900,000 (Morris). In these cases the cells are abnormally small, otherwise the cell volume would exceed that of the whole blood.

Decrease of red corpuscles, or *oligocythemia*. Red corpuscles and hemoglobin are commonly decreased together, although usually not to the same extent.

Oligocythemia occurs in all but the mildest symptomatic anemias. The blood-count varies from near the normal in moderate cases down to 1,500,000 in very severe cases. There is always a decrease of red cells in chlorosis, but it is often slight, and is relatively less than the decrease of hemoglobin. Leukemia gives a decided oligocythemia, the average count being about 3,000,000. The greatest loss of red cells occurs in pernicious anemia, where counts below 1,000,000 are not uncommon.

Method of Counting.—Although simple in principle, accurate counting of blood-corpuscles involves a technic which is acquired only after considerable practice. Exact and fairly rapid work is demanded. Before beginning, one should familiarize himself with the instrument and its ruling, and should read the directions carefully, giving especial attention to sources of error. It is likewise an advantage to practice sucking the diluting fluid into the pipet and

stopping it at a predetermined height. In our class work we have found Emerson's plan satisfactory: after students think they have acquired the technic they are required to count their own red corpuscles at the same hour upon successive days until the difference between the lowest and highest of five successive counts falls below 200,000 cells. Only by rigid adherence to such a plan can a student realize his inaccuracies.

When purchasing a hemacytometer it is well, despite the additional cost, to secure one which has been tested by the United States Bureau of Standards. The Bureau of Standards mark is placed upon each piece of such instruments. In the case of the cover-glass it is placed on the side, which is always to be used uppermost.

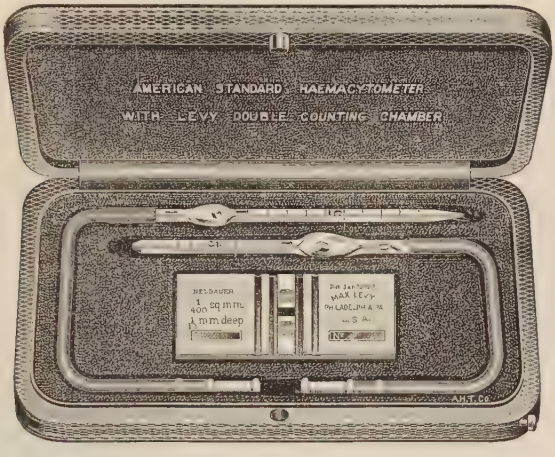


FIG. 110.—Hemacytometer,*consisting of a counting chamber, a cover-glass, and two pipets for diluting the blood.

The **hemacytometer** consists of two pipets for diluting the blood and a counting chamber (Fig. 110). The rubber tubes which come with the pipets are often too short and too flexible and should be replaced. For this purpose nothing is so good as a rubber catheter.

The counting chambers are of two general types:

(*a*) The *open* or *Bürker type* (Figs. 111 and 112), now almost universally adopted, consists of a heavy glass slide, upon the middle third of which are fixed three narrow parallel platforms extending across the slide. The middle platform or "floor piece" is exactly 0.1 mm. lower than the two others. Upon it is ruled a square millimeter subdivided into 400 small squares. Each fifth row of small squares is subdivided

by an extra line for convenience in keeping track of the areas to be counted (Fig. 114). Surrounding this are eight other square millimeters which are subdivided differently by Neubauer, Zappert, and others (Fig. 120). Only the central square of 400 small squares is used in counting red corpuscles

In the "improved Neubauer ruling" now used on Levy and Levy-Hausser counting chambers, the extra line mentioned above as subdi-

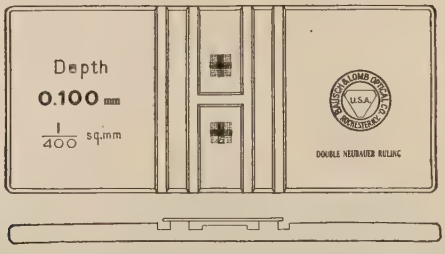


FIG. 111.—A new "open" counting chamber of excellent type. It is made in one piece and the surfaces of the platforms are highly polished, making it possible to obtain Newton's bands as a criterion of proper application of the cover-glass. The lower figure shows the chamber in cross-section with cover-glass in place.

viding each fifth row of small squares is omitted, and the 400 small squares are divided into twenty-five groups of sixteen squares each by a "split" boundary line (Fig. 113).

Some counting chambers of this type are supplied with cover-glass clamps, which is a decided advantage. In the "double counting chamber"

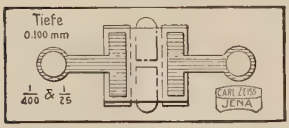


FIG. 112.—Bürker's open counting chamber with spring clamps holding the cover-glass in place.

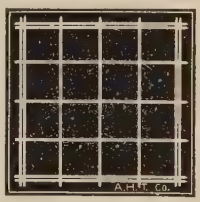


FIG. 113.—Group of 16 small squares of "improved Neubauer ruling," showing split boundary lines.

(Fig. 112) the middle platform is divided into two by a transverse groove and a ruling is scored upon each.

(*b*) The *closed* or *Thoma-Zeiss counting chamber*, which has been almost wholly superseded by the type just described, has a square platform in the center of which is a circular opening. In this opening is set a small circular disk in such manner that it is surrounded by a "moat," and that its surface is exactly 0.1 mm. below the surface of the square

platform. Upon this disk is scored one or another of the rulings mentioned above.

A thick cover-glass, ground perfectly plane, accompanies the counting chamber. Ordinary cover-glasses are of uneven surface, and should not be used with this instrument. For use with objectives of short working distance, heavy cover-glasses can be obtained with a flat-bottomed excavation or "well" in the center. This combines the advantages of a thin cover with the rigidity of a thick one.

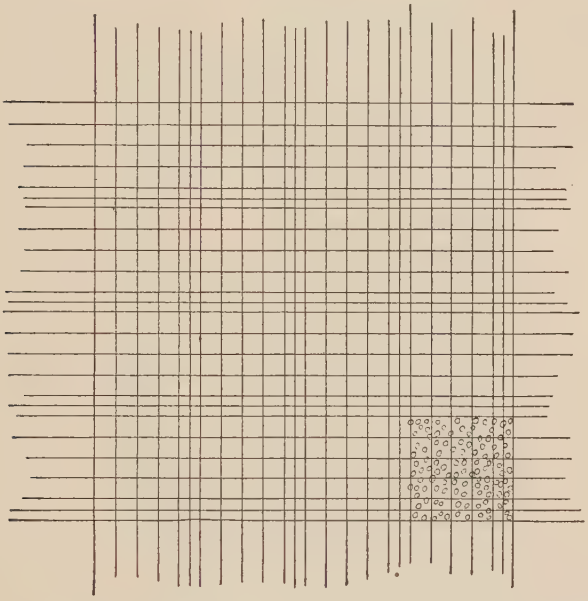


FIG. 114.—Central square millimeter of the ruling of the blood-counting chamber. It is subdivided into 400 small squares and each fifth row of small squares is indicated by a median line for convenience in locating areas to be counted. This is the standard ruling for counting red corpuscles, which are shown in the lower right corner.

It is evident that, when the cover-glass is in place upon the platform of either type of counting chamber (Fig. 111) there is a space exactly 0.1 mm. thick between it and the ruled platform or disk; and that, therefore, each square millimeter of the ruling forms the base of a space holding exactly 0.1 cubic millimeter.

Filling the Pipet.—To count the red corpuscles use the pipet with 101 engraved above the bulb. It must be clean and dry. Puncture the skin, wipe off the first drop of blood, and fill the pipet from the second, sucking the blood to the mark 0.5 or 1, according to the dilution desired. While doing this, hold the pipet horizontally at nearly right angles to

the line of vision, so that the exact height of the column may be easily seen. The side of the tip should rest against the skin, but the end must be free. Air-bubbles will enter with the blood if the drop is too small or if the tip is not kept immersed. Should the blood go slightly beyond the mark, draw it back by touching the tip of the pipet to a moistened handkerchief. Quickly wipe off the blood adhering to the tip, plunge it into the diluting fluid, and suck the fluid up to the mark 101, slightly rotating the pipet meanwhile. At this stage it is best to hold the pipet nearly vertically in order to avoid inclusion of a large air-bubble in the bulb. This dilutes the blood 1 : 200 or 1 : 100, according to the amount of blood taken. Except in cases of severe anemia, a dilution of 1 : 200 is preferable. Close the ends of the pipet with the fingers, and shake vigorously until the blood and diluting fluid are well mixed, keeping the pipet horizontal meanwhile. It should not be shaken in the direction of the long axis. One to two minutes' shaking is usually sufficient.

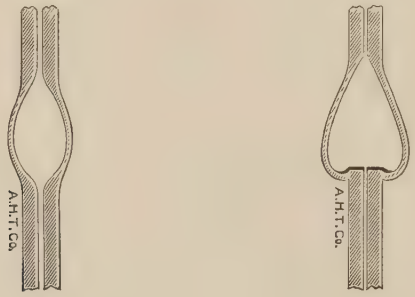


FIG. 115.—Cross-section of bulb and part of stem of diluting pipets: A, Original Thoma pipet; B, Trenner automatic pipet. In the latter, blood fills the stem by capillarity and automatically stops when it reaches the bulb.

To fill the Trenner automatic pipet (Fig. 115) draw the blood by suction into the capillary portion until it is nearly full. Discontinue the suction and, if the pipet be held nearly horizontally, the blood will continue to rise to the end of the capillary and will automatically stop there. The diluting fluid then is immediately drawn in by suction as described above for the Thoma pipet.

When it is not convenient to count the corpuscles at once, place a heavy rubber band around the pipet so as to close the ends, inserting a small piece of rubber-cloth or other tough, non-absorbent material, if necessary, to prevent the tip from punching through the rubber. It may be kept thus for twenty-four hours or longer. Still better closure devices can be purchased at slight cost. One of these is shown in Figure 116.

Charging the Counting Chamber.—When ready to make the count, clean the counting chamber and cover-glass, and place a sheet of paper over them to keep off dust.

(*a*) *Open* (*Bürker Type*) *Counting Chamber.*—Adjust the cover-glass and clamp it in place if the counting chamber is supplied with clamps. If the cover is properly adjusted and the slide be viewed obliquely, faint concentric lines of the prismatic colors—Newton's bands—can be seen between the cover and the platforms upon which it rests. They indicate that the two surfaces are in close apposition. If they do not appear at once, slight pressure upon the cover may bring them out. Failure to obtain them is usually due to dirty slide or cover—both must be perfectly clean and *free from dust.* Some counting chambers have a mat

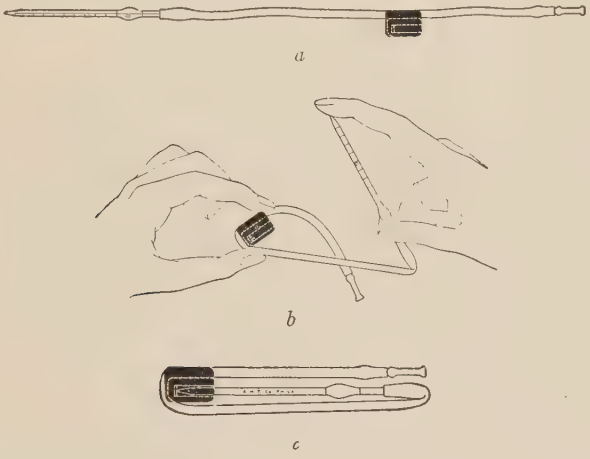


FIG. 116.—New closure device for diluting pipets, showing manner of application.

surface which makes it impossible to bring out Newton's bands, but is said to have certain compensating advantages.

When the cover is properly adjusted, mix the fluid in the pipet thoroughly by shaking for about a minute. Now quickly blow 2 or 3 drops from the pipet, wipe off its tip, and, holding the pipet in an inclined position, touch its tip to the angle between the edge of the cover-glass and one of the projecting ends of the floor piece. The fluid will run under the cover by capillary attraction. Care must be exercised to use just enough fluid to fill the space beneath the cover. This is especially important when the instrument lacks cover-glass clamps, since an excess of fluid will tend to raise the cover appreciably and thus increase the cell count. In a properly filled counting chamber the fluid nearly or quite fills the space beneath the cover, none has run over into the moat,

and there are no bubbles. If these conditions are not met the work must be done over again.

(*b*) *Closed* (*Thoma-Zeiss Type*) *Counting Chamber.*—Mix the fluid in the pipet thoroughly by shaking, blow 2 or 3 drops from the pipet, wipe off its tip, and then place a small drop (the proper size can be learned only by experience) upon the disk of the counting chamber. Adjust the cover immediately. Hold it by diagonal corners above the drop of fluid, so that a third corner touches the slide and rests upon the edge of the platform. Place a finger upon this corner, and, by raising the finger, allow the cover to fall quickly into place. If the cover be properly adjusted, Newton's bands can be seen between it and the platform when the slide is viewed obliquely. The drop placed upon the disk must be of such size that, when the cover is adjusted, it nearly or quite covers the disk, and that none of it runs over into the "moat." There should be no bubbles upon the ruled area.

The following is an easier method of applying the cover: Place a drop of fluid upon the ruled disk. The size of the drop is of no great consequence, if only it be large enough. Immediately place the cover-glass flat upon one side of the platform with its edge close to the drop of fluid, and hold it firmly down with the two index-fingers, or with the index-finger and middle finger of the right hand. Now slide it firmly and quickly into place. If the drop of fluid be too large, the excess will be caught on the top of the cover. A moderately thin cover is necessary. Should the cover not be applied immediately after the fluid is placed upon the disk the corpuscles will settle and thus increase the count.

Counting.—Allow the corpuscles to settle for a few minutes, and then examine with a low power to see that they are evenly distributed. If they are not *evenly distributed over the whole disk*, the counting chamber must be cleaned and a new drop placed in it.

Inexperienced workers often have difficulty in locating the ruled area. On this account the Levy and Levy-Hausser counting chambers now have a deeply ruled line extending from the end of the platform to the ruled area. This prominent "finding line" is quickly located with either high or low objectives.

Probably the most satisfactory objective for counting is the 8 mm. or the 4 mm. with long working distance. To understand the principle of counting it is necessary to remember that the square millimeter (400 small squares) represents a capacity of 0.1 cu. mm. Find the number of corpuscles in the square millimeter, multiply by 10 to find the number in 1 cu. mm. of the diluted blood, and finally, by the dilution, to find the number in 1 cu. mm. of undiluted blood. Instead of

actually counting all the corpuscles it is customary to count those in only a limited number of small squares, and from this to calculate the number in the square millimeter. Nearly every worker has his own method of doing this. The essential thing is to adopt a method and adhere to it. The following plan, applicable to 1 : 200 dilution, is widely used: Count the red cells in a group of 25 small squares in each corner of the square millimeter, making 100 squares in all, multiply by 8, and add three ciphers.

In practice a convenient procedure is as follows: *With a dilution of 1 : 200, count the cells in 80 small squares, and to the sum add 4 ciphers;*

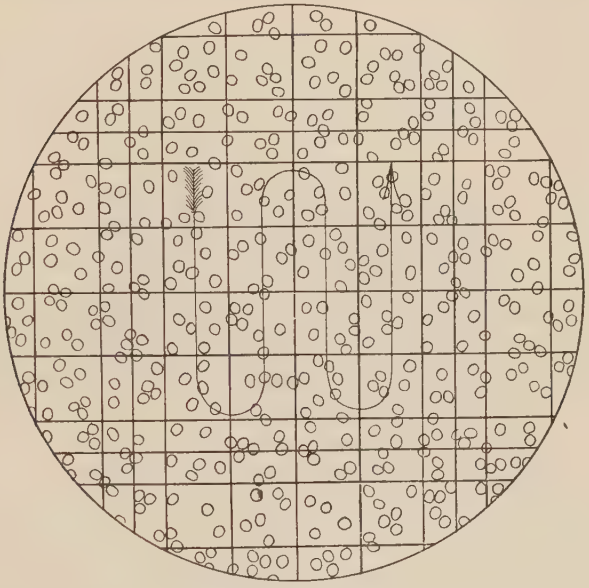


Fig. 117.—Appearance of microscopic field in counting red corpuscles. The arrow indicates the 16 squares to be counted in the short method described in the text.

with dilution of 1 : 100, count 40 small squares and add 4 ciphers. Thus, if with 1 : 200 dilution, 450 corpuscles were counted in 80 squares, the total count would be 4,500,000 for each cubic millimeter. This method is sufficiently accurate for most clinical purposes, provided the corpuscles are evenly distributed and 2 drops from the pipet be counted. It is convenient to count five blocks of sixteen small squares (Fig. 117), one such block in each corner of the square millimeter, and one block near the center.

In order to avoid confusion in counting cells which lie upon the border-lines, the following rule is generally adopted: *Corpuscles which*

touch the upper and left sides should be counted as if within the squares, those touching the lower and right sides, as outside; or vice versa.

Diluting Fluids.—The most widely used are Hayem's and Toisson's. Both of these have high specific gravities, so that, when well mixed, the corpuscles do not separate quickly. Toisson's fluid is perhaps the better for beginners, because it is colored and can easily be seen as it is drawn into the pipet. It stains the nuclei of leukocytes blue, but this is no real advantage. It must be filtered frequently because of the ready growth of fungi in it. Hayem's fluid is to be preferred for routine work. For convenience in filling pipets the fluids should be kept in small wide-mouth bottles.

Hayem's Fluid		Toisson's Fluid	
Mercuric chlorid	0.5	Sodium chlorid	1.0
Sodium sulphate	5.0	Sodium sulphate	8.0
Sodium chlorid	1.0	Glycerin	30.0
Distilled water	200.0	Distilled water	160.0
		Methyl-violet, 5 B to give a strong purple color.	

Sources of Error.—The most common sources of error in making a blood-count are:

(*a*) Inaccurate dilution, usually from faulty technic, occasionally from inaccurately graduated pipets. Only an instrument of standard make can be relied upon, and it is best to purchase one which has been tested by the United States Bureau of Standards.

(*b*) Too slow manipulation, allowing a little of the blood to coagulate and remain in the capillary portion of the pipet.

(*c*) Inaccuracy in depth of counting chamber usually due to imperfect application of the cover-glass, but sometimes to faulty manufacture or to softening of the cement by alcohol or heat. A cemented slide should not be cleaned with alcohol or left to lie in the warm sunshine.

(*d*) Uneven distribution of the corpuscles. This results when the blood has partially coagulated, when it is not thoroughly mixed with the diluting fluid, when the cover-glass is moved after the counting chamber is filled, or when the suspension overflows into the moat. The last may sometimes reduce the count as much as 1,000,000.

(*e*) The presence of yeasts, which may be mistaken for corpuscles, in the diluting fluid.

Cleaning the Instrument.—The instrument should be cleaned immediately after using, and the counting chamber and cover must be cleaned again just before use.

Transfer the rubber tube to the small end of the pipet and draw through it, successively, water, alcohol, ether, and air; or water, acetone,

and air. This can be done with the mouth, but it is much better to use a rubber bulb or suction filter pump. When the mouth is used, the moisture of the breath will condense upon the interor of the pipet unless the fluids be shaken, and not blown, out. If blood has coagulated in the pipet—which happens when the work is done too slowly—dislodge the clot with a horsehair (which can be obtained from the hair-cloth interlining of a coat), never with a wire, and clean with strong sulphuric acid, or let the pipet stand overnight in a test-tube of the acid. Even if the pipet does not become clogged, it should be occasionally cleaned in this way. When the etched graduations on the pipets become dim, they can be renewed by rubbing with a wax pencil.

Wash the counting chamber and the cover with water and dry them with clean soft linen. Alcohol may be used to clean the latter, but never the former, unless it be of the new non-cement type, although a handkerchief *slightly* moistened with alcohol may be used to wipe off the surface of the ruled disk and the platform.

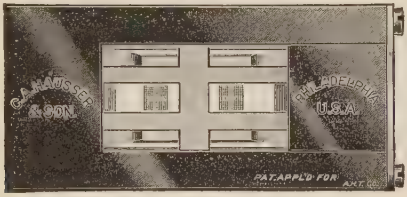


FIG. 118.—Hausser's counting chamber in bakelite holder. The cover-glass and clamps are not shown.

Hausser's counting chamber (Fig. 118) is a new form, consisting of a bakelite slide in which is placed a small removable counting chamber which is made entirely in one piece and thus avoids the troublesome loosening of parts which have been cemented in place with balsam. Counting chambers with various rulings, of both single and double form, and of different depths for counting blood-platelets, blood-corpuscles, and the cells of spinal fluid, can be had and are interchangeable in the bakelite holder. All are of the "open" type. The instrument is equipped with cover-glass clips which, however, are not shown in the illustration.

V. COLOR INDEX

This is an expression which indicates the amount of hemoglobin in each red corpuscle compared with the normal amount. For example, a color index of 1 indicates that each corpuscle contains the normal amount of hemoglobin; of 0.5, that each contains one-half the normal.

The color index is very significant in chlorosis and pernicious anemia. In the former it is usually much decreased; in the latter, generally much increased. In hemorrhagic anemia it is low and may be very low, suggesting chlorosis. In symptomatic anemia it is moderately diminished.

To obtain the color index, divide the *percentage* of hemoglobin by the *percentage* of corpuscles. For this purpose the normal number of corpuscles (100 per cent.) is assumed to be 5,000,000 for each cu. mm. The percentage of corpuscles may be found by multiplying the first two figures of the red corpuscle count by 2. This simple method holds good for all counts of 1,000,000 or more. Thus, a count of 2,500,000 is 50 per cent. of the normal. If, then, the hemoglobin has been estimated at 40 per cent. divide 40 (the percentage of hemoglobin) by 50 (the percentage of corpuscles). This gives $\frac{4}{5}$, or 0.8, as the color index.

From what has already been said regarding the variations in hemoglobin instruments, and of the impossibility of fixing a normal standard for either red cells or hemoglobin which is applicable to all ages and in all localities, it would appear that color-index calculations, as above described, have little value. They do, however, yield information of great value in many cases of anemia, provided that the hemoglobinometer used has been standardized to give an average reading of 100 per cent. upon the bloods of healthy adults with red corpuscle counts of 5,000,000 in each cubic millimeter (p. 236). In childhood, from about the sixth month to the fifteenth year, the color index is normally low, since the hemoglobin values fall disproportionately during this period of life. It is probable that the normal corpuscles are saturated with hemoglobin, and therefore that a high color index must be dependent on increased size of the corpuscles. A low index, on the other hand, may be due either to small size of the red cells or to lowered concentration of hemoglobin in their substance.

VI. VOLUME INDEX

The term "volume index" was introduced by Capps to express the average size of the red cells of an individual compared with their normal size. It is the quotient obtained by dividing the *volume* of red corpuscles (expressed in percentage of the normal) by the *number* of red corpuscles, also expressed in percentage of the normal. This means of expressing the average size of the red cells

gives more accurate information than does measuring them under the microscope.

The volume index more or less closely parallels the color index, but is more dependable and more significant. A volume index above 1 is practically constant in pernicious anemia and is now regarded as one of the most important signs of the disease. The following are averages of the examinations reported by Larrabee:

	Red corpuscles for each cubic millimeter.	Hemoglobin per cent. by Sahli instrument.	Color index.	Volume index.
Normal males	5,267,250	103.0	0.98	1.007
Normal females	4,968,667	106.0	1.06	1.001
Primary pernicious anemia	1,712,166	50.0	1.47	1.270
Secondary anemia	3,737,160	61.0	0.81	0.790
Chlorosis	3,205,000	34.5	0.55	0.695

Method.—The red cells are counted and the percentage of red cells calculated as for the color index.

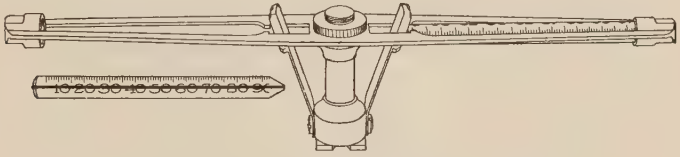


FIG. 119.—Daland hematocrit for use with the centrifuge.

The volume percentage is obtained with the hematocrit as follows: Fill the hematocrit tubes (Fig. 119) with blood. If held horizontally they readily fill by capillary attraction. Before coagulation takes place insert them in the frame and centrifugalize for three minutes at about 8000 to 10,000 revolutions a minute. The red cells collect at the bottom and, normally, make up nearly one-half of the total column of blood. Multiply the height of the layer of red cells (as indicated by the graduations upon the side of the tube) by 2 to obtain the volume percentage.

More recently a larger amount of blood, taken from a vein and mixed with oxalate as described for blood chemistry (p. 333) was used. This is placed to the 100 mark in the graduated tube of the Sahli hemoglobinometer. The blood is centrifugalized at high speed for one-half hour, or longer, until the maximum amount of packing of corpuscles has taken place; the volume of the red corpuscles is noted; the percentage relation to the normal is calculated by dividing this volume by the normal volume and multiplying by 100. The normal volume of

the packed cells is 0.48 of the total volume; that is, the corpuscles of the blood of normal persons would stand at 48 on the Sahli scale. Example: Suppose the packed cells of the blood under examination reached to the 29 mark, then the volume percentage would be 29/48 or 60 per cent. If the red cell count is 50 per cent. of the normal (2,500,000 for each cu. mm.) then 60/50, or 1.2 is the volume index.

A similar method which requires a still larger quantity of blood has been described by Haden and is now much used. A graduated 15 c.c. centrifuge tube is filled exactly to the 2 c.c. mark with 1.6 per cent. sodium oxalate solution, and 10 c.c. of blood is added exactly to the 12 c.c. mark. The tube is inverted to oxalate the blood. The cells are packed to the maximum amount, normal blood packing to 4.6 c.c. or 4.8 c.c. The value for normal blood should be determined with each centrifuge. The subsequent calculations are similar to those described in the preceding paragraph.

VII. ENUMERATION OF LEUKOCYTES

The normal number of leukocytes varies from 5000 to 10,000 per cubic millimeter of blood. The number is larger in robust individuals than in poorly nourished ones, and, if disease be excluded, may be taken as a rough index of the individual's nutrition. It rises appreciably in the afternoon, regardless of the intake of food. Sabin finds the highest count on any day to be approximately twice the lowest count on the same day (the highest usually between 1 and 5 P. M.), and she also finds an hourly rhythm in the count. These variations depend on the neutrophiles chiefly. Since it is well to have a definite standard, 7500 is generally adopted as the normal for the adult. With children the number is somewhat greater, averaging about 10,000 to 12,000 in infants and somewhat below 10,000 in older children. Leukocyte counts have much less diagnostic value in young children than in adults, since the blood of children reacts much more markedly and often in an atypical manner.

Decrease in Number of Leukocytes

Decrease in number of leukocytes, or *leukopenia*, is not important. Counts of 3500 to 4000 per cubic millimeter are not uncommon in persons who are poorly nourished, although not actually sick. The infectious diseases in which leukocytosis is absent (p. 258) often cause a slight and sometimes a marked decrease in number of leukocytes, owing probably to toxic inhibition of bone-marrow ac-

tivity. Leukocytes occasionally fall below 1000 for each cubic millimeter. Chlorosis may produce leukopenia, as also pernicious anemia, which usually gives it in contrast to the secondary anemias, which are frequently accompanied by leukocytosis. Leukocyte counts are, therefore, of some aid in the differential diagnosis of these conditions.

In contrast to the normal digestive leukocytosis (p. 257), Widal has called attention to a digestive leukopenia, along with accelerated coagulation of the blood and a fall in blood-pressure, in persons suffering from hepatic disease. This so-called "hemoclastic crisis" is induced by 200 c.c. of milk given upon an empty stomach, the maximal response occurring in about forty-five to fifty minutes. At one time it promised to be useful as a test of hepatic insufficiency, but is now little regarded excepting as an interesting phenomenon.

Increase in Number of Leukocytes

Increase in number of leukocytes is common and of great importance. It may be considered under two heads:

A. Increase of leukocytes due to chemotaxis and stimulation of the blood-making organs, or *leukocytosis*. The increase affects one or more of the normal varieties.

B. Increase of leukocytes due to *leukemia*. Normal varieties are increased, but the characteristic feature is the appearance of great numbers of abnormal cells.

The former may be classed as a *transient*, the latter as a *permanent*, increase.

A. Leukocytosis

This term is variously used. By some it is applied to any increase in number of leukocytes; by others it is restricted to increase of the polymorphonuclear neutrophilic variety. As has been indicated, it is here taken to mean a transient increase in number of leukocytes, that is, one caused by chemotaxis and stimulation of the blood-producing structures, in contrast to the permanent increase caused by leukemia.

By *chemotaxis* is meant that property of certain agents by which they attract or repel living cells—positive chemotaxis and negative chemotaxis respectively. An excellent illustration is the accumulation of leukocytes at the site of inflammation, owing to the positively chemotactic influence of bacteria and their products. A great many

agents possess the power of attracting leukocytes into the general circulation. Among these are many bacteria and certain organic and inorganic poisons.

Chemotaxis alone will not explain the continuance of leukocytosis for more than a short time. It is probable that substances which are positively chemotactic also stimulate the blood-producing organs to increased formation of leukocytes, and in at least one form of leukocytosis such stimulation apparently plays the chief part.

As will be seen later, there are several varieties of leukocytes in normal blood, and most chemotactic agents attract only one variety, and either repel or do not influence the others. It practically never happens that all are increased in the same proportion. The most satisfactory classification of leukocytoses is, therefore, based upon the type of leukocyte chiefly affected.

Theoretically, there should be a subdivision for each variety of leukocyte, for example, neutrophilic leukocytosis, lymphocytic leukocytosis, eosinophilic leukocytosis, endothelial leukocytosis, and so forth. Practically, however, only two of these, polymorphonuclear leukocytosis and lymphocytic leukocytosis, need be considered under the head of Leukocytosis. Increase in number of the other leukocytes will be considered when the individual cells are described (pp. 287–297). They are present in the blood in such small numbers normally that even a marked increase scarcely affects the total leukocyte count; and, besides, substances which attract them into the circulation frequently repel the polymorphonuclears, so that the total number of leukocytes may actually be decreased.

The polymorphonuclear neutrophils are capable of active ameboid motion, and are by far the most numerous of the leukocytes. Lymphocytes are about one-third as numerous and have little independent motion. As one would, therefore, expect, marked differences exist between the two types of leukocytosis; neutrophilic leukocytosis is more or less acute, coming on quickly and often reaching high degree; whereas lymphocytic leukocytosis is more chronic, comes on more slowly, and is seldom so marked.

1. Polymorphonuclear Neutrophilic Leukocytosis.—Neutrophilic leukocytosis may be either physiologic or pathologic. A count of 20,000 would be considered a marked leukocytosis: of 30,000, high; above 50,000, extremely high.

(1) **Physiologic Neutrophilic Leukocytosis.**—This is never very marked, the count seldom exceeding 12,000 or 14,000 in each cubic millimeter. It may occur: (*a*) In the newborn, as high as 18,000 in the first few days of life; (*b*) in pregnancy during the ninth month, most marked in primiparæ; (*c*) during labor, averaging about 18,000 in primiparæ, much less in multiparæ, and subsiding during the first few days of the puerperium; (*d*) during digestion, and (*e*) after cold baths. There is moderate leukocytosis in the moribund state, occasionally reaching 20,000; this is commonly classed as physiologic, but is probably due mainly to terminal infection.

The increase in these conditions is not limited to the neutrophils. Lymphocytes are likewise increased in varying degrees, most markedly in the newborn.

In view of the normal rise in the afternoon and of the leukocytosis of digestion, which usually increases the leukocytes by about 30 per cent., the hour at which a leukocyte count is made should always be recorded. Digestive leukocytosis is most marked three to five hours after a hearty meal rich in protein, especially when such a meal follows a long fast. It is absent in pregnancy and when leukocytosis from any other cause exists. It is usually absent in cancer of the stomach, a fact which may be of some help in the diagnosis of this condition, but repeated examinations and careful technic are essential. The absence of digestive leukocytosis in liver disease (Widal's test of liver function) has been mentioned on page 255.

(2) **Pathologic Neutrophilic Leukocytosis.**—In general, the response of the leukocytes to chemotaxis is a conservative process. It has been compared to the gathering of soldiers to destroy an invader. This is accomplished partly by means of phagocytosis—actual ingestion of the enemy—and partly by means of chemical substances which the leukocytes produce.

In those diseases in which leukocytosis is the rule the degree of leukocytosis depends upon two factors: the *severity of the infection* and the *resistance of the individual.* A well-marked leukocytosis usually indicates good resistance. A mild degree means that the body is not reacting well, or else that the infection is too slight to call forth much resistance. Leukocytosis may be absent altogether when the infection is extremely mild, or when it is so severe as to

overwhelm the organism before it can react. When leukocytosis is marked, a sudden fall in the count may be the first warning of a fatal issue. These facts are especially true of pneumonia, diphtheria, and abdominal inflammations, in which conditions the degree of leukocytosis is of considerable prognostic value.

The classification here given follows Cabot in the main:

(*a*) *Infectious and Inflammatory.*—The majority of infectious diseases produce leukocytosis. The degree varies with the site and nature of the disease, the virulence of the infection, the resistance of the individual, and the presence of complications, hence no definite figures can be given for any particular disease. However, the counts which are to be expected in typical cases of the more important infectious diseases which produce leukocytosis are somewhat as follows: Pneumonia, 20,000 to 30,000, a few counts over 100,000 having been recorded; scarlet fever, 20,000 to 30,000; diphtheria, 15,000 to 25,000; erysipelas, 20,000 to 25,000; Asiatic cholera, 25,000 to 30,000; meningococcus meningitis, 20,000 to 30,000; tuberculous meningitis, 10,000 to 20,000; acute articular rheumatism, 10,000 to 15,000.

The most important infectious diseases *which do not cause leukocytosis* are influenza; measles; German measles; malaria; mumps; tuberculosis, except when invading the meninges or when complicated by mixed infection; and typhoid and paratyphoid fevers, in which leukocytosis indicates an inflammatory complication. In the recent pandemic of influenza uncomplicated cases generally showed low normal leukocyte counts, averaging 5000 to 6000, with a tendency to subnormal counts on the first day or two and with the lymphocytes at a high normal level or slightly increased. In an occasional case the count was 12,000 to 15,000. With the onset of pneumonia a definite leukocytosis was usual.

All inflammatory and suppurative processes cause leukocytosis, except when slight or well walled off. This is particularly marked in infections with staphylococci, streptococci, and pneumococci. Appendicitis has been studied with especial care in this connection, and the conclusions now generally accepted probably hold good for most acute intra-abdominal inflammations. A marked leukocytosis (20,000 or more) nearly always indicates abscess, peritonitis, or gangrene, even though the clinical signs be slight. Absence of or mild leukocytosis indicates a mild process, or else an overwhelm-

ingly severe one; and operation may safely be postponed unless the abdominal signs are very marked. On the other hand, no matter how low the count, an increasing leukocytosis—counts being made hourly—indicates a spreading process and demands operation, regardless of other symptoms.

Leukocyte counts alone are often disappointing in infectious and inflammatory conditions, but are of much greater value when considered in connection with the percentage of neutrophils (p. 292). *Every total leukocyte count should be accompanied by a differential count.*

(*b*) *Malignant Disease.*—Leukocytosis occurs in about one-half of the cases of malignant disease. In many instances it is probably independent of any secondary infection, since it occurs in both ulcerative and non-ulcerative cases. It seems to be more common in sarcoma than in carcinoma. Very large counts are rarely noted.

(*c*) *Postoperative.*—The number of leukocytes rises moderately after operations irrespective of infection, reaching a maximum in about six hours. The degree depends upon the severity of the operation, amount of tissue damaged, loss of blood, and possibly upon the anesthetic used, chloroform probably inducing a greater reaction than does ether.

(*d*) *Posthemorrhagic.*—Moderate leukocytosis follows hemorrhage and disappears in two to four days. This is especially true of hemorrhage into the serous cavities which nearly always increases the leukocyte count 150 to 300 per cent. within ten hours. In cases of ruptured tubal pregnancy with hemorrhage into the peritoneal cavity the count usually reaches 18,000 to 30,000.

(*e*) *Toxic.*—This is a rather obscure class, which includes gout, chronic nephritis, acute yellow atrophy of the liver, ptomain-poisoning, prolonged chloroform narcosis, and quinin-poisoning. Leukocytosis may or may not occur in these conditions, and is not important.

(*f*) *Drugs.*—This also is an unimportant class. Most tonics and stomachics and many other drugs produce a slight leukocytosis. A moderate leukocytosis may also occur as a result of prolonged chloroform or ether anesthesia.

2. Lymphocytic Leukocytosis.—This is characterized by an increase in the total leukocyte count, accompanied by an increase

in the percentage of lymphocytes. The word "lymphocytosis" is often used in the same sense. It is better, however, to use the latter as referring to any increase in the absolute number of lymphocytes, without regard to the total count, since an absolute increase in number of lymphocytes is frequently accompanied by a normal or subnormal leukocyte count, owing to loss of neutrophils.

Lymphocytic leukocytosis is probably due more to stimulation of blood-making organs than to chemotaxis. It is less common, and is rarely so marked as a neutrophilic leukocytosis. When marked, the blood cannot be distinguished from that of lymphatic leukemia.

A marked or high lymphocytic leukocytosis occurs in pertussis and in no other important condition. It is said to appear early in the catarrhal stage and to reach its maximum at the height of the paroxysmal stage, after which it gradually subsides. In thirty well-marked cases studied by Schneider the average leukocyte count was 19,000 in the first week, rising to about 27,000 in the third. His lowest counts in the first week were 12,600, and in the third 16,800. Leukocyte counts would, therefore, seem to have great value in diagnosis, but in our experience they have often been disappointing, since in many mild cases the count does not rise above what may be regarded as a high normal for children before the characteristic whoop begins.

There is slight or moderate lymphocytic leukocytosis in other diseases of childhood, as rickets, scurvy, and especially hereditary syphilis, where the blood-picture may at times approach that of pertussis. It must be borne in mind in this connection that lymphocytes are normally more abundant in the blood of children than in that of adults.

Within the past few years a number of cases of "acute infectious mononucleosis" with glandular enlargement and fever have been reported by Cabot, Sprunt and Evans, Longcope,[1] Downey and McKinlay,[2] and others. The striking feature of the blood has been a marked leukocytosis with preponderance of mononuclear cells which appear to be lymphocytes or cells derived from them.

[1] For details see Longcope, W. T.: Infectious Mononucleosis (Glandular Fever), with a Report of 10 Cases, Amer. Jour. Med. Sci., vol. 164, p. 781, December, 1922.

[2] Downey, H., and McKinlay, C. A.: Acute Lymphadenosis Compared with Acute Lymphatic Leukemia, Arch. Int. Med., vol. 32, p. 82, July, 1923.

B. Leukemia

This is an idiopathic disease of the blood-making organs which is accompanied by an enormous increase in number of leukocytes. The leukocyte count sometimes reaches 1,000,000 per cubic millimeter, and leukemia is always to be suspected when it exceeds 50,000. Lower counts do not, however, exclude it. The subject is more fully discussed later (p. 383).

Method of Counting Leukocytes

The leukocytes are counted with the hemacytometer already described (p. 243). Numerous modifications of the original ruling have been introduced, notably the Türck, the Zappert-Ewing, and

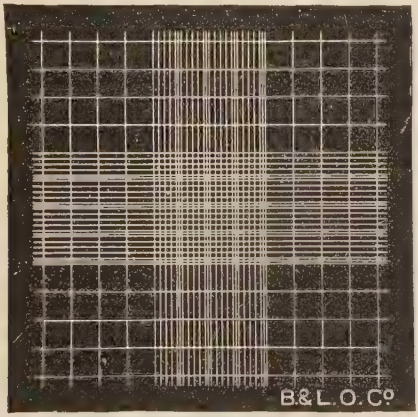


Fig. 120.—Neubauer ruling for counting chamber (× 15).

the Neubauer (Fig. 120), which give a ruled area of 9 sq. mm., the center having the ruling which is used for counting the red corpuscles. Of these, the Neubauer may be especially commended. Some of them were originally devised for counting the leukocytes in the same dilution with the red corpuscles. The two kinds of cell are easily distinguished, especially when Toisson's diluting fluid is used. The red cells are counted in the central portion in the usual manner, after which all the leukocytes in the whole area of 9 sq. mm. are counted, and the number in a cubic millimeter of undiluted blood is then calculated.

Although less convenient, it is more accurate to count the leukocytes separately, with less dilution of the blood, as described in

the following paragraphs. With care the range of error is easily kept within 5 per cent., although in hurried office work it is generally much greater.

Technic.—A larger drop of blood is required than for counting the erythrocytes, and more care in filling the pipet, since the bore is considerably larger than that of the "red" pipet. Boggs has suggested a device (Fig. 121) which enables one to draw in the blood more slowly and hence more accurately. He cuts the rubber tube and inserts a Wright "throttle." This consists of a section of glass tubing within which a capillary tube drawn out to a fine thread is cemented with

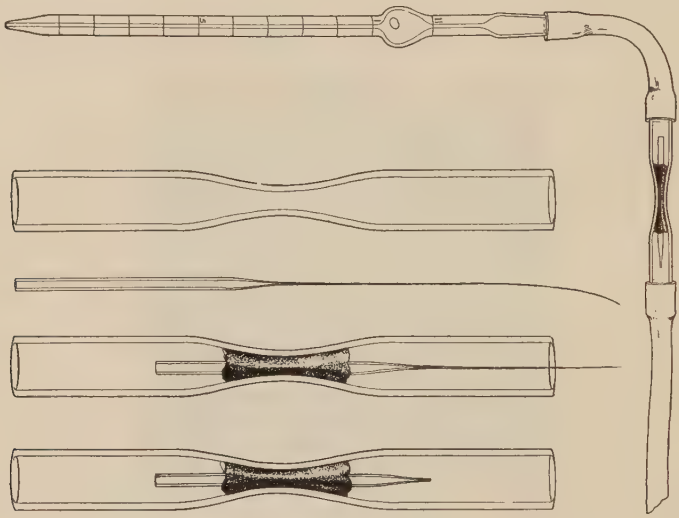


FIG. 121.—Boggs' "throttle control" for blood-counting pipet, and enlarged diagram showing construction of the throttle.

sealing wax. After sealing in place the tip is broken off with forceps, so that upon gentle suction it will just allow air to pass.

Use the pipet with 11 engraved above the bulb.[1] Suck the blood to the mark 0.5 or 1, and the diluting fluid to the mark 11. This gives a dilution of 1 : 20 or 1 : 10, respectively. The dilution of 1 : 20 is easier to make. If the blood should accidentally go much above the 0.5 mark, draw it to 0.6 and multiply the final count by 5/6. Mix well by shaking in all directions except in the long axis of the pipet; blow out 2 or 3 drops, place a drop in the counting chamber, and charge the counting chamber as already described (p. 247).

[1] In some cases of leukemia with very high count it may be necessary to use the "red" pipet with dilution of 1 : 100.

Examine with a low power to see that the cells are evenly distributed. Count with the 16-mm. objective and a high eye-piece, or with the long-focus 4 mm. and a low eye-piece. An 8-mm. objective will be found very satisfactory for this purpose. As one gains experience one will rely more upon the lower powers. A square millimeter may be included in the field of the 16-mm. objective with a medium ocular.

Count all the leukocytes in several square millimeters and find the average per square millimeter. Multiply this by 10 to find the number in 1 cu. mm. of diluted blood, and by the dilution to find the number

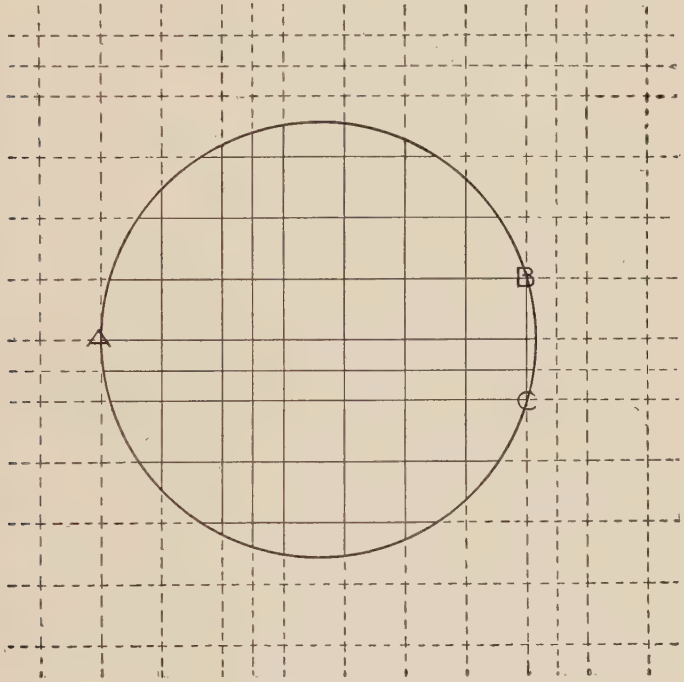


FIG. 122.—Size of field required in counting leukocytes as described in the text.

in a cubic millimeter of undiluted blood. In every case at least 200 leukocytes must be counted as a basis for calculation, and it is much better to count 500. With Neubauer and similar rulings and a dilution of 1 in 20, a convenient plan is the following: With a low-power objective count the leukocytes in the square millimeters at each of the four corners of the ruled area and multiply by 50. This should be repeated upon a second and a third slide and the average taken.

In routine work Todd's modification of the "circle" method is very satisfactory. It requires a 4-mm. objective, and is, therefore, espe-

cially desirable for beginners, who are usually unable accurately to identify leukocytes with a lower power. The student is frequently confused by particles of dirt, remains of red cells, and yeast cells which are prone to grow in the diluting fluid. Draw out the sliding tube of the microscope until the field of vision is such as shown in Figure 122. One side of the field is tangent to one of the ruled lines, A, while the opposite side just cuts the corners, B and C, of the seventh squares in the rows above and below the diameter line. When once adjusted, a scratch is made upon the draw-tube, so that for future counts the tube has only to be drawn out to the mark. The area of this microscopic field is 0.1 sq. mm. With a dilution of 1 : 20, count the leukocytes in 20 such fields upon different parts of the disk without regard to the ruled lines, and to their sum add two ciphers. With dilution of 1 : 10, count 10 such fields, and add two ciphers. Thus, with 1 : 10 dilution, if 150 leukocytes were counted in 10 fields, the leukocyte count would be 15,000 for each cubic millimeter. To compensate for possible unevenness of distribution, it is best to count a row of fields horizontally and a row vertically across the disk. This method is applicable to any degree of dilution of the blood, and is simple to remember: *one always counts a number of fields equal to the number of times the blood has been diluted, and adds two ciphers.* The count should be repeated upon several slides and the average taken.

It is sometimes impossible to obtain the proper size of field with the objectives and eye-pieces at hand. In such case place a cardboard or stiff paper disk with a circular opening upon the diaphragm of the eye-piece, and adjust the size of the field by drawing out the tube. The circular opening can be cut with a sharp cork-borer.

Diluting Fluids.—The diluting fluid should dissolve the red corpuscles so that they will not obscure the leukocytes. The simplest fluid is a 1 per cent. solution of acetic acid. More satisfactory is the following: glacial acetic acid, 1 c.c.; 1 per cent. aqueous solution of gentian-violet, 1 c.c.; distilled water, 100 c.c. These solutions must be filtered frequently to remove yeasts and molds.

VIII. ENUMERATION OF BLOOD-PLATELETS

The normal number of blood-platelets, when counted by the direct method described below, ranges from 250,000 to 350,000 for each cubic millimeter of blood. They are intimately connected with the process of coagulation and this appears to be their chief function. The precise rôle which they play is not, however, entirely clear. They are apparently the chief source of the prothrombin of the blood-plasma; they probably furnish at least part

of the thromboplastic substance of the tissues; and, after coagulation has taken place, they undoubtedly serve to bring about retraction of the clot, since the rapidity and degree of retraction vary directly with the number of platelets present. In general, whenever from any cause the platelets fall below 60,000 for each cubic millimeter of blood, a hemorrhagic tendency becomes evident.

Physiologic variations in number of platelets are marked. Thus, the number increases as one ascends to a higher altitude, and is higher in winter than in summer. There are unexplained variations from day to day; hence a single abnormal count should not be taken to indicate a pathologic condition. In disease, variations are often extremely great. An increase may be due to increased number or increased activity of the bone-marrow giant-cells (megakaryocytes) which are the mother-cells of the platelets. A decrease is more important. It may be referable to injury or destruction of the megakaryocytes, or to destruction of platelets in the circulation. There is no means of recognizing increased destruction of platelets as there is in the case of red corpuscles. The normal life of the platelets is estimated (Lee and Minot) at about four days. Even when platelets are present in normal numbers they may be functionally defective.

Upon the clinical side the following facts seem to be established:

(*a*) In acute infectious diseases the number is usually normal or subnormal. In diphtheria, especially, the count may fall to a very low level with development of a tendency to hemorrhage. As a rule, the platelets increase during convalescence.

(*b*) In secondary anemia, especially posthemorrhagic, platelets are generally increased, although sometimes decreased. In pernicious anemia they are nearly always greatly diminished, and an increase would question the diagnosis of this condition.

(*c*) They are decreased in chronic lymphatic leukemia; variable but usually greatly increased in chronic myelogenous leukemia, much decreased in acute leukemia of either form.

(*d*) They are somewhat increased in tuberculosis.

(*e*) Platelet counts are of great value in differentiating the hemorrhagic diseases. Both hemophilia and purpura hæmorrhagica are caused by disturbances of the platelets, but may be sharply differentiated. In hemophilia platelets are normal in number, but defective in function, bringing about coagulation very slowly.

The clot, though delayed, is well formed and has normal retractile power. In purpura hæmorrhagica, upon the other hand, platelets are functionally normal, but greatly reduced in numbers, ranging from about 40,000 to 75,000 for each cubic millimeter in mild cases, and down to 15,000 or below in severe cases. Coagulation time is about normal, but the platelets are too few to cause normal retraction of the clot.

Blood-platelets are difficult to count, owing to the rapidity with which they disintegrate and to their strong tendency to adhere to any foreign body and to each other. The unavoidable error is greater than in counting red corpuscles or leukocytes, but is negligible in practice, because only very great variations in the count have clinical significance. It is often possible to recognize a great loss of platelets from a careful inspection of stained films, provided these are made evenly and very quickly in order to avoid clumping.

Method of Counting Platelets.—Many methods have been proposed. Of these, the direct method, which employs the hemacytometer already described, is the most practicable for clinical purposes, although the counts are regularly lower than are yielded by some of the indirect methods.

Rapid work is necessary in order to prevent clumping of the platelets. The diluting fluid is drawn to near the 1 mark in the "red" pipet, blood from a freely bleeding puncture is drawn exactly to the 0.5 mark, and finally the diluting fluid is quickly drawn to the 101 mark. This gives a blood-dilution of 1 in 200. The blood and diluting fluid are immediately mixed by shaking for about two minutes; the counting chamber is filled at once, and ten minutes are allowed for the corpuscles to settle before counting is begun. The count is made with the high dry objective and a high ocular (10×) in the manner described for red corpuscles.

Diluting Fluid of Wright and Kinnicutt.—Platelets appear as rounded, lilac-colored bodies; red corpuscles are decolorized, appearing only as shadows; leukocytes are stained.

Aqueous solution brilliant cresyl blue (1 : 300)	2 parts
Aqueous solution potassium cyanid (1 : 1400)	3 parts

These two solutions are kept in separate bottles and mixed and filtered just before using. The cresyl blue solution is permanent, but molds have a tendency to grow in it. The cyanid solution deteriorates after about ten days.

Diluting Fluid of Rees and Ecker.—This solution preserves the red corpuscles, which may be counted in the same specimen.

Sodium citrate, 3.8 per cent. aqueous solution	100.0 c.c.
Formaldehyd, 40 per cent. solution	0.2 "
Brilliant cresyl blue	0.1 gm.

Diluting Fluid of Leake and Guy.—This fluid has been found especially satisfactory. It does not destroy the red corpuscles.

Distilled water	94.0 c.c.
Formalin, 40 per cent. solution	6.0 "
Sodium oxalate	1.6 gm.
Crystal violet	0.05 gm.

The fluid is warmed, filtered, and preserved in stoppered bottles. It keeps well.

IX. STUDY OF STAINED BLOOD

A. Making and Staining Blood-films

1. Spreading the Film.—*Properly spread films are essential to accurate and pleasant work.* They more than compensate for the time spent in learning to make them. There are certain requisites for success with any method: (*a*) The slides and covers must be perfectly clean: thorough washing with soap and water, rubbing with alcohol and drying on a clean handkerchief will usually suffice; (*b*) the drop of blood must not be too large; (*c*) the work must be done quickly, before coagulation begins.

The blood is obtained from the finger-tip or the lobe of the ear, as for a blood-count; only a very small drop is required, usually about twice the size of a pinhead. The size of the drop largely determines the thickness of the film. The proper thickness will depend upon the purpose for which the film is made. For the structure of blood-cells and the malarial parasite it should be so thin that, throughout the greater part of the film, the red corpuscles lie in a single layer, close together but not overlapping. In our class work we insist that all films turned in for inspection meet this requirement. For routine differential counting of leukocytes a film in which the red cells are piled up somewhat is best because the leukocytes are more evenly distributed, and because the number of leukocytes in a given area is greatly increased and the tedium of counting is correspondingly lessened. The film must not, upon the

other hand, be so thick that identification of the various leukocytes becomes difficult. In some cases of severe anemia it is very difficult to make good films owing to the large proportion of plasma, which leads to slow drying, with consequent distortion of the red cells and the appearance of artifacts. To overcome this the films should be made very thin and dried quickly over a low flame.

Nearly all ordinary slides are slightly curved. In order that they may lie firmly upon the microscope stage without rocking, the blood film should be spread upon the convex side, which is recognized by laying the slide flat upon the table and twirling it rapidly by snapping the end with a finger. The side upon which it twirls the better is the convex side.

Ehrlich's Two Cover-glass Method.—This method is widely recommended, but considerable practice is required to get good results.

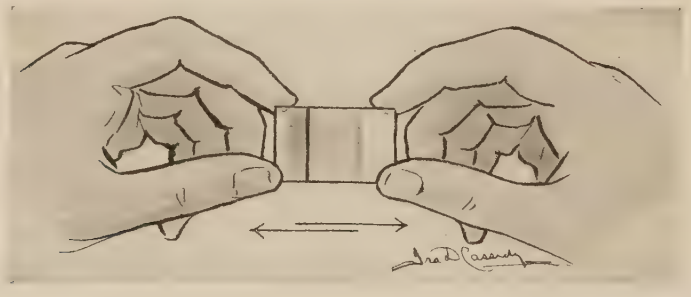


FIG. 123.—Spreading the film: two cover-glass method. It is better to place the top cover diagonally and to grasp it by opposite corners, and still better to handle both covers with forceps.

Touch a large cover-glass to the top of a small drop of blood, and place it, blood side down, upon another large cover-glass. If the drop be not too large, and the covers be perfectly clean, the blood will spread out in a very thin layer. Just as it stops spreading, before it begins to coagulate, pull the covers quickly but firmly apart on a plane parallel to their surfaces (Fig. 123). It is generally advised to handle the covers with forceps, since the moisture of the fingers may produce artifacts. The forceps must have a firm grasp.

This method is especially to be recommended for very accurate differential counts, since all the leukocytes in the drop will be found on the two covers, and thus the error due to unequal distribution can be excluded by counting all the leukocytes. One of the covers is usually much better spread than the other.

Slide-and-cover Method.—Beacom uses a slide and cover in a

manner similar to that described above for two cover-glasses. A small drop of blood from a puncture is taken upon a clean slide about $\frac{1}{2}$ inch from the end, and a clean thin cover-glass is quickly applied. As soon as the blood has ceased spreading the tips of two fingers are placed on the cover, and, with *the lightest possible pressure,* the cover is slid quickly along the slide, leaving a thin film of blood behind it. The method is much easier to learn than is the two-cover-glass method, gives more uniform films, and apparently gives more uniform distribution of leukocytes. All of the blood remains on the slide, none, or practically none, on the cover; hence the method is especially useful for very accurate differential counts, since every leukocyte in the drop of blood can be classified. Slow work, allowing partial coagulation, results in rupture of many of the leukocytes, as is also the case with the two-cover-glass method.

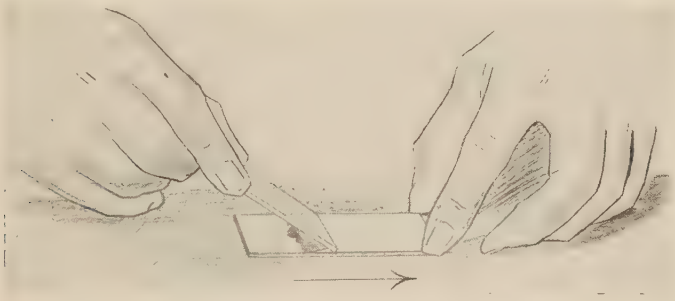


FIG. 124.—Spreading the film: two-slide method.

Two-slide Method.—Take a small drop of blood upon a clean slide about $\frac{3}{4}$ inch from the end, using care that the slide does not touch the skin. Place the end of a second slide against the surface of the first at an angle of 30 to 40 degrees, and draw it up against the drop of blood, which will immediately run across the end, filling the angle between the two slides. Now push the "spreader slide" back along the other in the manner indicated in Figure 124. The blood will follow. The thickness of the film can be regulated by changing the angle, by varying the pressure, and by using a smaller or larger drop of blood.

It is very easy by this method to make large, thin, even films which are especially useful for studying the red corpuscles and the malarial parasite. Their use for differential leukocyte counting is discussed on page 286.

The films may be allowed to dry in the air, or may be dried by gently warming high above a flame (where one can comfortably

hold the hand). Such films will keep for years, but for some stains they must not be more than a few weeks old. They must be kept away from flies—a fly can work havoc with a film in a few minutes. F. W. Lacy suggested an interesting novelty. After the film is made and dried, half of it is covered with a card and the other half is placed near the mouth and breathed on a number of times. The red corpuscles in this part are nearly or quite destroyed, leaving the leukocytes, which stand out distinctly when stained. The part of the film which has been protected from the breath stains in the usual way.

When slides are used the label can be written with a soft lead pencil directly on the blood-film, as was suggested by von Ezdorf.

2. Fixing the Film.—In general, films must be "fixed" before they are stained. Fixation may be accomplished by chemicals or by heat. *Those stains which are dissolved in methyl alcohol combine fixation with the staining process.*

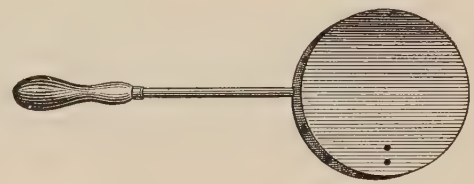


FIG. 125.—Kowarsky's plate for fixing blood (Klopstock and Kowarsky).

Chemical Fixation.—Soak the film one to two minutes in pure methyl alcohol or absolute ethyl alcohol, or fifteen minutes or longer in equal parts of absolute alcohol and ether. One minute in 1 per cent. solution of mercuric chlorid or in 1 per cent. formalin in alcohol is preferred by some, especially for the carbolthionin stain. The film must be well washed in water after mercuric chlorid fixation. Chemical fixation may precede hematoxylin-eosin and other simple stains.

Heat Fixation.—This may precede any of the methods which do not combine fixation with the staining process. The best method is to place the film in an oven, raise the temperature to 150° C., and allow to cool slowly. Without an oven, the proper degree of fixation is difficult to attain. Kowarsky has devised a small plate of two layers of copper (Fig. 125), upon which the films are placed together with a crystal of urea. The plate is heated over a flame until the urea melts, and is then set aside to cool. Some prefer to use slides and to place the crystal of urea directly upon the slide. This is said to give the proper

degree of fixation, but in Todd's experience the films have always been underheated. He obtains better results by use of tartaric acid crystals (melting-point 168°–170° C.). The plate, upon which have been placed the cover-glasses, film side down, and a crystal of the acid, is heated over a low flame until the crystal has completely melted. It should be held sufficiently high above the flame that the heating will require five to seven minutes. The covers are then removed. Freshly made films of normal blood should be allowed to remain upon the plate for a minute or two after heating has ceased. Fresh films require more heat than old ones, and normal blood more than the blood of pernicious anemia and leukemia.

Blood-films can be satisfactorily fixed for most purposes by covering with absolute alcohol, quickly dashing off the excess, and igniting the remainder.

3. Staining the Film.—The anilin dyes, which are extensively used in blood work, are of two general classes: basic dyes, of which methylene-blue is the type, and acid dyes, of which eosin is the type. Nuclei and certain other structures in the blood are stained by the basic dyes, and are hence called *basophilic*. Certain structures take up only acid dyes, and are called *acidophilic*, or *oxyphilic*, or *eosinophilic*. Certain other structures are stained by combinations of the two, and are called *neutrophilic*. Recognition of these staining properties marked the beginning of modern hematology.

(1) **Hematoxylin and Eosin.**—This method is most useful in studying eosinophilic cells and the structure of nuclei, hematoxylin being, in fact, one of our best nuclear stains. It may therefore be recommended for the Arneth count (p. 293). Red corpuscles are pink or red, all nuclei blue, eosinophilic granules bright red; neutrophilic granules and platelets are not stained. Neither polychromatophilia nor basophilic granular degeneration of the red cells are demonstrated.

1. Fix by heat or chemicals.
2. Stain with any standard hematoxylin solution until nuclei are well colored, usually three to five minutes.
3. Wash well with water.
4. Apply a weak aqueous or alcoholic solution of eosin (about 0.5 per cent.) for a minute or two.
5. Wash well in water, dry, and examine. If the eosin stains too deeply, longer washing ir water will usually remove some of the excess.

The hematoxylin will not wash out, but is rather intensified by washing in water.

The procedure may be simplified by mixing the hematoxylin and eosin. Such a mixture was much used before modern staining methods were introduced. Almost any of the standard hematoxylin solutions may be employed; to this is added enough of a saturated aqueous solution of eosin to color the reds properly while the hematoxylin is staining the nuclei. The combined stain keeps well. The fixed smear is immersed in the staining fluid for the required time, usually five to fifteen minutes, and is then rinsed, dried, and mounted.

(2) **Polychrome Methylene-blue-eosin Stains.**—These stains, outgrowths of the original time-consuming Romanowsky method, have largely displaced other blood-stains for clinical purposes. They may be recommended for all routine work. They stain differentially every normal and abnormal structure in the blood. Most of them are dissolved in methyl alcohol and combine the fixing with the staining process. Numerous methods of preparing and applying these stains have been devised, among the best known being Giemsa's, Wright's, Hastings', and Leishman's.

Wright's Stain.—This is one of the best and is the most widely used in this country. The practitioner will find it convenient to purchase the stain ready prepared, but, since much of the solution offered for sale is unsatisfactory, it is best to purchase the powder and dissolve it in methyl alcohol as needed. Most microscopic supply-houses carry it in stock. Wright's most recent directions for its preparation and use are as follows:

Preparation.—To a 0.5 per cent. aqueous solution of sodium bicarbonate add methylene-blue (B. X. or "medicinally pure") in the proportion of 1 gm. of the dye to each 100 c.c. of the solution. Heat the mixture in a steam sterilizer at 100° C. for one full hour, counting the time after the sterilizer has become thoroughly heated. The mixture is to be contained in a flask, or flasks, of such size and shape that it forms a layer not more than 6 cm. deep. After heating, allow the mixture to cool, placing the flask in cold water, if desired, and then filter it to remove the precipitate which has formed in it. It should, when cold, have a deep purplish-red color when viewed in a thin layer by transmitted yellowish artificial light. It does not show this color while it is warm.

To each 100 c.c. of the filtered mixture add 500 c.c. of a 0.1 per cent.

aqueous solution of "yellowish water-soluble" eosin and mix thoroughly. Collect the abundant precipitate, which immediately appears, on a filter. Owing to lack of uniformity in the dyes now obtainable it may be necessary to add more or less of the eosin to obtain a satisfactory precipitate. When the precipitate appears it may be recognized by placing a drop of the fluid upon filter-paper. Dry the precipitate thoroughly, dissolve it in methylic alcohol (Merck's "reagent") in the proportion of 0.1 gm. to 60 c.c. of the alcohol. In order to facilitate solution the precipitate is to be rubbed up with the alcohol in a porcelain dish or mortar with a spatula or pestle. This alcoholic solution of the precipitate is the staining fluid. We frequently find that freshly made solutions stain the red cells blue owing to slight alkalinity; such solutions usually work properly after a few months.

Application.—1. Without previous fixation cover the film with a noted quantity of the staining fluid by means of a medicine-dropper. There must be plenty of stain in order to avoid too great evaporation and consequent precipitation. When slides are used, the stain may be confined to the desired area by two heavy wax-pencil marks.

2. After one minute add to the staining fluid on the film the same quantity of distilled water by means of a second medicine-dropper. This may be done by counting drops. The drops of water are about twice as large as the drops of stain, but this rarely does any harm and is often an advantage. The quantity of the fluid on the preparation must not be so large that some of it runs off. Allow the mixture to remain for three to six minutes, according to the intensity of the staining desired. A longer period of staining may produce a precipitate. Eosinophilic granules are best brought out by a short period of staining.

3. Wash the preparation in water for thirty seconds, or until the thinner portions of the film become yellow or pink in color. The preparation should be flooded with water while the stain is still upon it. If the stain is poured off before rinsing, the scum tends to settle upon the blood-film, where it clings in spite of subsequent washing.

4. Dry, best by waving high above a flame, and mount in balsam. When the films are on slides examine directly with the oil-immersion objective.

The stain is more conveniently applied upon cover-glasses than upon slides. Films much more than a month old do not stain well, red cells and most other structures taking a slate blue color. In some localities ordinary tap-water will answer both for diluting the stain and for washing the film; in others, distilled water must be used. The difficulty here is probably that the tap-water is acid in

reaction. This causes the nuclei to stain too palely. Other causes of pale nuclei are addition of too much or too little water and the development of formic acid from the methyl alcohol of the staining fluid.

When properly applied Wright's stain gives the following picture (Frontispiece and Plate VII): erythrocytes, yellow or pink; nuclei, various shades of purple; neutrophilic granules, reddish lilac, sometimes pink; eosinophilic granules, bright red; basophilic granules of leukocytes and degenerated red corpuscles, very dark bluish purple; blood-platelets, dark lilac; bacteria, blue. The cytoplasm of lymphocytes is generally robin's-egg blue; that of the endothelial leukocytes has a faint bluish tinge. Malarial parasites stain characteristically: the cytoplasm, sky-blue; the chromatin, purplish red. These colors are not invariable: two films stained from the same bottle sometimes differ greatly. *In general, a preparation is satisfactory when both nuclei and neutrophilic granules are distinct, regardless of their color, and when the film is free from precipitated dye.* In addition, it is desirable, but not essential, that the red corpuscles show a clear pink or yellowish pink; they should not be blue. The colors are prone to fade if the preparation is mounted in a poor quality of balsam or exposed much to the light.

Failure to get satisfactory results with the polychrome methylene-blue-eosin stains, when they are properly used, may be due to imperfect polychroming of the powder; but is most frequently a question of incorrect reaction of the staining fluid. When the solution is too acid the red corpuscles stain bright red and the nuclei of the leukocytes are pale sky-blue or even colorless. When it is too alkaline the red corpuscles stain deep slate-blue and there is little differentiation of colors. The reaction of the solution is determined partly by that of the powder, when, as in the case with Wright's stain, its reaction is not accurately adjusted; but it depends to a still greater degree upon the methyl alcohol, which is prone to develop formic acid as a result of oxidation upon standing. A given powder may afford perfect results when dissolved in methyl alcohol from a freshly opened bottle, and give poor results when dissolved in the same lot of alcohol after it has stood for some months exposed to the air. Deterioration of old solutions is largely due to the same cause. It is possible to correct imperfectly acting fluids with traces of potassium hydrate or acetic acid, as suggested by

Peebles and Harlow, but this is not very satisfactory. We find it better to keep on hand all fluids which have become too acid for use and to mix them as required with the fresh solutions, which are generally too alkaline when made with alcohol from a newly opened bottle. However, such solutions will correct themselves in time. Pathologic bloods will sometimes not stain well with solutions which are correct for normal blood.

Other Useful Blood-stains.—While Wright's stain suffices for most clinical work and is to be recommended if only one blood-stain is to be used, certain others demand brief mention.

1. **Giemsa's Stain.**—This widely used stain is probably the best modification of the Romanowsky stain for blood parasites and other protoza, and is also very satisfactory as a routine blood-stain. It consists of:

Azur II-eosin	3.0 gm.
Azur II	0.8 "
Glycerin (Merck, C. P.)	250.0 "
Methyl alcohol (Kahlbaum I or Merck's reagent)	250.0 "

The solution is expensive to make and is best purchased ready prepared. Blood films are fixed in methyl alcohol, and are then immmersed for twenty minutes or longer in a freshly prepared mixture of 1 c.c. of stain and 10 c.c. distilled water. In order to prevent precipitates falling upon them, the slides or covers should be placed upon edge in the stain. Satisfactory results may also usually be obtained by placing about 30 drops of distilled water upon the fixed film, adding 3 drops of Giemsa's stain, mixing, and allowing it to act for fifteen or twenty minutes.

The use of this stain for *Treponema pallidum* is described later (p. 558).

2. **Pappenheim's Panoptic Method.**—In order to combine the advantages of the several stains Pappenheim recommended the following procedure: Stain for one minute with the May-Grünwald stain; add an equal quantity of water; after one minute pour off the fluid and stain fifteen minutes with the diluted Giemsa solution. The May-Grünwald stain is the same as Jenner's. Wright's stain, diluted with an equal quantity of water, may be substituted for the Giemsa solution, but the time of staining should then not exceed five minutes.

It is difficult to see that slides stained in this way offer any advantages over good Wright or Giemsa preparations.

3. **Jenner's Stain.**—Jenner's eosinate of methylene-blue, dissolved in methyl alcohol, brings out leukocytic granules well, and is, therefore,

especially useful for differential counting. It stains nuclei poorly, and is much inferior to Wright's stain for the malarial parasite since it does not give the so-called "Romanowsky staining."

It may be purchased in solution, in the form of tablets, or as a powder, 0.5 gm. of which is to be dissolved in 100 c.c. neutral absolute methyl alcohol. The unfixed blood-film is covered with the staining solution, and after three to five minutes is rinsed with water, dried in the air, and mounted.

4. **Carbol-thionin** is especially useful for the study of basophilic granular degeneration of the red cells. The method is described on page 270. Nuclei, malarial parasites, and basophilic granules are brought out sharply. Polychromatophilia is also evident. Fixation may be by alcohol-formalin (p. 270) or saturated solution of mercuric chlorid.

5. **Pappenheim's pyronin-methyl green** (p. 698) can be used as a blood-stain, and is very satisfactory for study of the red cells and of the lymphocytes and for demonstration of Doehle's inclusion bodies (p. 294). All nuclei are blue to reddish purple; basophilic granules, cytoplasm of lymphocytes, and inclusion bodies red. Polychromatophilia is well demonstrated, the affected cells taking more or less of the red color. Heat fixation is probably best.

B. Study of Stained Films

It has been said with much truth that an intelligent study of the stained film, together with an estimation of hemoglobin, will yield 90 per cent. of all the diagnostic information obtainable from a blood examination. The stained films furnish the best means of studying the morphology of the blood and blood parasites, and, to the experienced, they give a fair idea of the amount of hemoglobin and the number of red and white corpuscles. An oil-immersion objective is required.

1. Erythrocytes.—Normally, the red corpuscles are acidophilic. The colors which they take with different stains have been described. When not crowded together, they appear as circular, homogeneous disks, of nearly uniform size, averaging 7.8 μ in diameter (Fig. 126). The center of each is somewhat paler than the periphery. Red cells are apt to be crenated when the film has dried too slowly. Four or five cases are on record in which the majority of the red corpuscles were markedly elongated or elliptical in healthy individuals, probably an hereditary anomaly.

Pathologically, red corpuscles vary in hemoglobin content, size and shape, staining properties, and structure.

Description of Plate V

Abnormal red-corpuscles. All drawn from actual specimens and all stained with Wright's stain except where noted. × 1000 (1 mm. = 1 μ).

Fig. 1.—Variations in size, shape, and hemoglobin content; from cases of pernicious anemia and chlorosis.

Fig. 2.—Polychromatophilia and basophilic granular degeneration; from cases of lead-poisoning and pernicious anemia.

Fig. 3.—Normoblasts, reticulated red cells, and one microblast. The top row represents stages in the development of the normoblast. The two reticulated red cells are stained with brilliant cresyl blue.

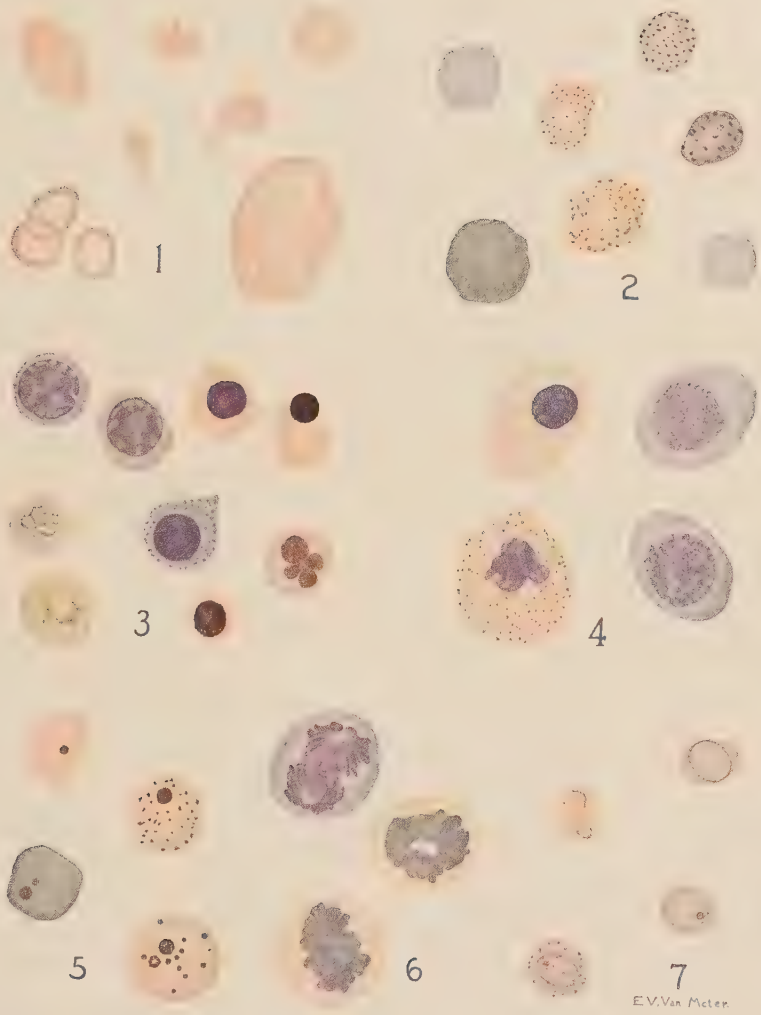
Fig. 4.—Megaloblasts from cases of pernicious anemia. Two show polychromatophilia and fairly typical nuclei, two have condensed nuclei, and one of these has basophilic cytoplasmic granules.

Fig. 5.—Nuclear particles or "Howell-Jolly bodies." One cell also shows basophilic granular degeneration.

Fig. 6.—Mitotic figures, two from myelogenous leukemia, one with polychromatophilic cytoplasm, from von Jaksch's anemia. The last was stained with Leishman's stain.

Fig. 7.—Cabot's ring bodies, from a case of von Jaksch's anemia. Two cells also contain nuclear particles and one shows basophilic granular degeneration. Leishman's stain.

PLATE V



(1) **Hemoglobin Content.**—The depth of staining furnishes a rough guide to the amount of hemoglobin in the corpuscles, that is, to the color index. When hemoglobin is diminished the central pale

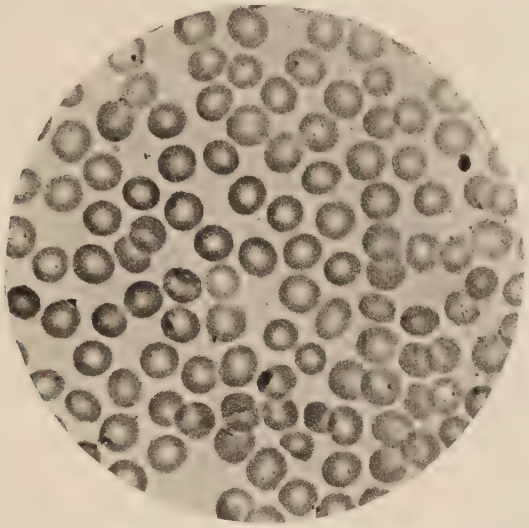


FIG. 126.—Red corpuscles of normal blood. Wright's stain (photograph, × 750).

area becomes larger and paler. This condition is known as *achromia* (Fig. 127). Usually the periphery retains a fairly deep color, so that the cells become mere rings, the so-called "pessary forms." These are most common in chlorosis. In pernicious anemia, upon

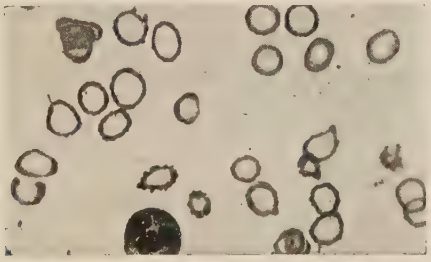


FIG. 127.—Red blood-corpuscles showing deficient hemoglobin (achromia). From a well-marked case of chlorosis. Wright's stain (photograph, × 750).

the other hand, as a result of the high color index, many of the red corpuscles may stain deeply and lack the pale center entirely.

(2) **Variations in Size and Shape** (Plate V, Fig. 1).—The cells may be abnormally small (called *microcytes*, 5 μ or less in diameter); abnormally large (*macrocytes*, 10–12 μ), or extremely large

(*megalocytes*, 12–25 μ). Abnormal variation in size is called *anisocytosis*. Many of the larger cells exhibit rounded or irregular, sharply or indefinitely outlined colorless areas, "endoglobular degeneration" (Fig. 128). This should not be confused with the highly refractile, colorless spots so frequently seen when the film has dried slowly, especially in the anemias.

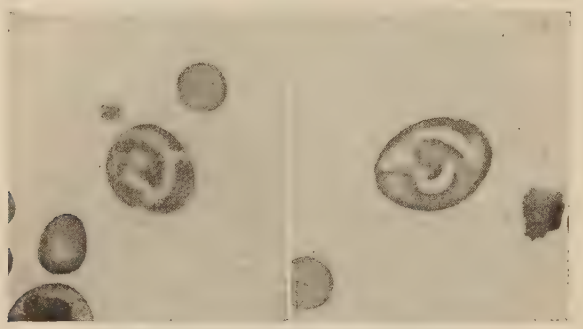


FIG. 128.—Two large red corpuscles (macrocytes) showing endoglobular degeneration (photographs, × 1000).

Variation in shape is often very marked. Oval, pyriform, caudate, saddle-shaped, and club-shaped corpuscles are common (Fig. 129). They are called *poikilocytes*, and their presence is spoken of as poikilocytosis.

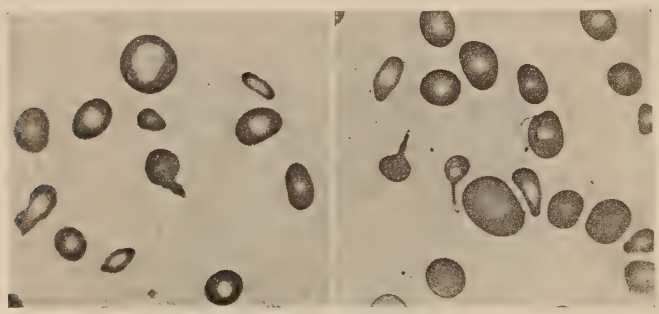


FIG. 129.—Red corpuscles showing variations in size and shape, from a case of pernicious anemia (photographs, × 750).

Red corpuscles which vary from the normal in size and shape are present in most anemias, and in the severer grades are often very numerous. Irregularities are particularly conspicuous in leukemia and pernicious anemia, where, in some instances, a normal erythrocyte is the exception. In pernicious anemia there is a decided tendency to large-size and oval forms, and megalocytes are

rarely found in any other condition. So constant is this in typical cases that a mere glance at a few fields often justifies the report "looks like pernicious anemia."

(3) **Variations in Staining Properties** (Plate V, Fig. 2).—These include polychromatophilia, basophilic granular degeneration, and malarial stippling. With exception of polychromatophilia they are probably degenerative changes.

(*a*) *Polychromatophilia*.—The affected corpuscles partially lose their normal affinity for acid stains and take the basic stain to greater or less degree. Wright's stain gives such cells a faint bluish tinge when the condition is mild, and a rather deep blue when severe. Sometimes only part of a cell is affected. A few polychromatophilic corpuscles can be found in marked symptomatic anemias. They occur most abundantly in malaria, leukemia, and pernicious anemia.

Polychromatophilia has been variously interpreted. It is thought by many to be evidence of youth in a cell, and hence to indicate an attempt at blood regeneration. There are probably several forms referable to different causes.

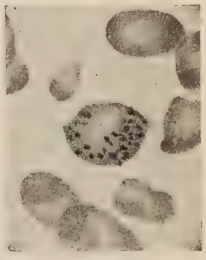


FIG. 130.—Red blood-corpuscle showing basophilic granular degeneration with large granules. Wright's stain (photograph, × 1000).

(*b*) *Basophilic Granular Degeneration* (*Degeneration of Grawitz, Basophilic Stippling*).—This is characterized by the presence, within the corpuscle, of irregular basophilic granules, which vary in size from scarcely visible points to granules nearly as large as those of basophilic leukocytes (Fig. 130). The number present in a red cell commonly varies in inverse ratio to their size. They stain deep blue with carbol-thionin or Wright's stain. The cell containing them may stain normally in other respects, or it may exhibit polychromatophilia. Polychromatophilic cells generally contain the smaller granules, which may be so fine that the cell appears dusted with them.

Numerous cells showing this degeneration are commonly found in chronic lead-poisoning, of which they were at one time thought to be pathognomonic. They can probably be found in every case with clinical symptoms and in some severe cases are present in nearly every microscopic field. Except in this disease the degeneration indicates a serious blood condition. It occurs in well-marked

cases of pernicious anemia and leukemia, and, much less commonly, in very severe symptomatic anemias.

(*c*) *Malarial Stippling.*—This term has been applied to the finely granular appearance often seen in red corpuscles which harbor tertian malarial parasites (Plates VI and VII). It was formerly classed with the degeneration just described, but is undoubtedly distinct. Not all stains will show it. With Wright's stain it can be brought out by staining longer and washing less than for the ordinary blood-stain. The minute granules, "Schüffner's granules," stain purplish red. They are sometimes so numerous as almost to hide the parasite.

(4) **Variations in Structure.**—The most important is the presence of a nucleus (Plate V, Figs. 3–7). Nucleated red corpuscles, or *erythroblasts*, are classed according to their size: *microblasts*,

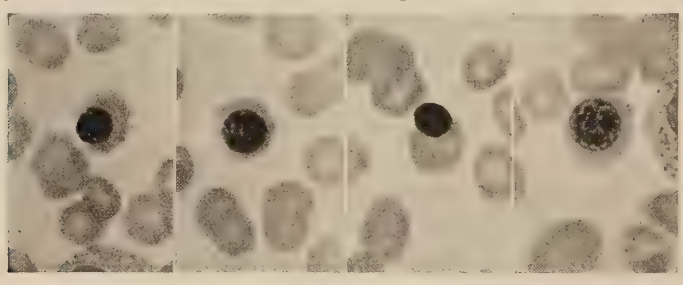


FIG. 131.—Normoblasts from cases of secondary anemia and leukemia. The next to the last is oldest, the last is youngest of the series (photographs, × 1000).

5 μ or less in diameter; *normoblasts*, 5 to 10 μ; and *megaloblasts*, above 11 μ.

Microblasts and **normoblasts** contain one, rarely two, small, round, sharply defined nuclei whose structure is decidedly different from that of the nuclei of leukocytes. As a rule they are the most deeply stained nuclei to be seen in the blood-film, being approached in this respect only by the smaller lymphocytes. Young normoblasts are large, often exceeding 11 μ. Their nuclei are relatively large and their chromatin is arranged in a more or less reticular manner with rather clean-cut open spaces. Not infrequently the openings are arranged at the periphery and, with the chromatin bars between them, suggest a wheel with broad spokes. Mitoses are not uncommon in leukemia and pernicious anemia. The older normoblasts are smaller. Their nuclei, also, are smaller and more dense, some being entirely homogeneous and very deeply stained

(pyknotic nuclei). These last are apt to be located eccentrically, and sometimes appear as if in process of extrusion from the cell. These characteristics are shown in Figure 131. It is important to distinguish the younger from the older cells. As a result of degenerative changes the nuclei may be irregular in shape (Fig. 132), clover-leaf forms being common; or they may be completely broken

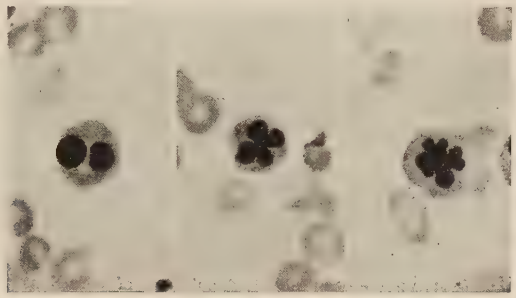


FIG. 132.—Normoblasts with irregular and fragmented nuclei. Wright's stain (photographs, × 1000).

up into fragments—the so-called **nuclear particles** or Howell-Jolly bodies—of which all but one or two may have disappeared from the cell. These nuclear particles are smooth, round, deeply stained bodies, not likely to be mistaken for the irregularly-shaped granules of basophilic degeneration (Fig. 133).

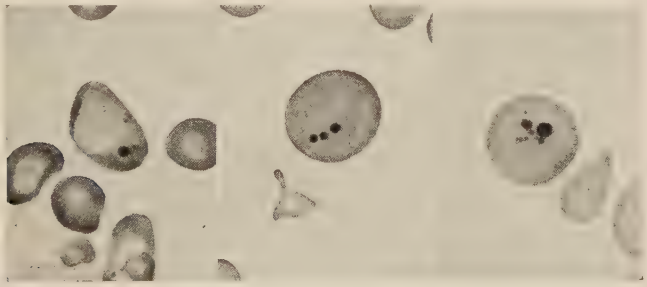


FIG. 133.—Nuclear particles or Howell-Jolly bodies in red corpuscles. From a case of pernicious anemia. Wright's stain (photographs, × 1000).

The **megaloblast** is probably a distinct cell, not merely a larger size of the normoblast. In the typical megaloblast the nucleus is characteristic. This is large, oval, and rather palely staining, and it has a more delicate chromatin network with larger and more numerous openings than has the nucleus of the normoblast (Plate V and Fig. 134). Sometimes it appears as if made up of coarse gran-

ules. As the megaloblast grows older the nucleus becomes smaller (Fig. 135) and more solidly stained and ultimately shows evidences of degeneration (pyknosis, karyorrhexis). At the same time the entire cell becomes smaller and the cytoplasm shows less tendency to polychromatophilia.

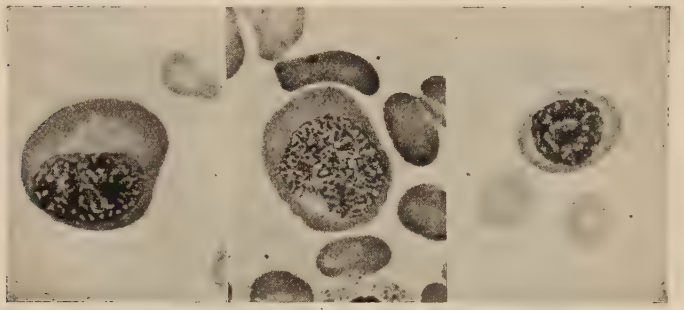


FIG. 134.—Megaloblasts showing typical nuclei; from cases of pernicious anemia. Wright's stain (photographs, × 1000).

The recognition of megaloblasts is important, but is not always easy unless the nucleus is typical. Some workers base the distinction from normoblasts upon size of nucleus, requiring this to be larger than a normal red corpuscle if the cell is to be regarded as a megaloblast. Others consider only the size of the cell, regarding as a megaloblast any nucleated red cell over 11 μ in diameter. Neither

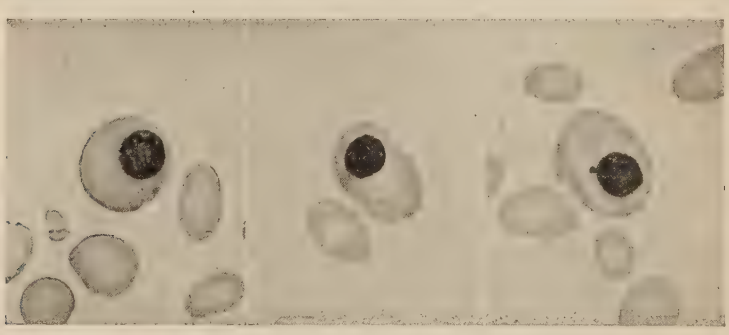


FIG. 135.—Small (aged) megaloblasts with condensed nuclei (photographs, × 1000).

of these rules nor the two together will serve in every case. The exceptions will include, on the one hand, certain old megaloblasts with small condensed nuclei, and, upon the other, the very young normoblasts whose diameter may exceed 12 μ and whose nuclei may be larger than a normal red corpuscle. At times one finds cells which must be classed as intermediates.

Young nucleated red cells, especially megaloblasts, are prone to exhibit polychromatophilia. In some cells the cytoplasm is so blue and shows so little of its characteristic smooth texture that it is difficult to recognize the cell as an erythrocyte except by the character of the nucleus. Such cells are often mistaken for lymphocytes

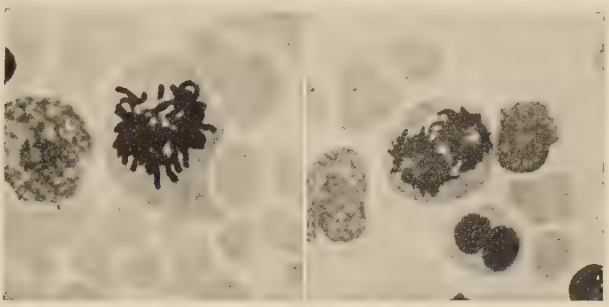


FIG. 136.—Two megaloblasts in the process of mitosis. Mitotic figures are often seen in the blood especially in leukemia, but are rarely so definite as these (photographs, × 1000).

or for Türck's irritation leukocytes, an error which careful observation of the nucleus even with the low power would usually prevent.

Cabot's ring bodies are ring- or figure-of-8-shaped structures (Fig. 137) which have been observed in certain of the red cells in pernicious anemia, lead-poisoning, and lymphatic leukemia. They

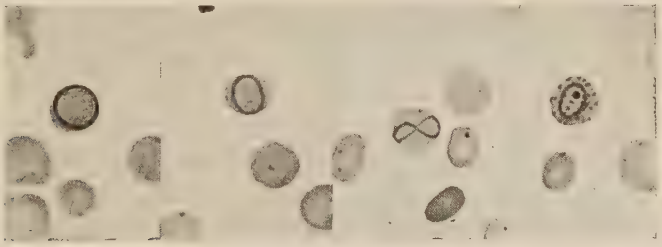


FIG. 137.—Cabot's ring bodies in red blood-corpuscles from a case of von Jaksch's anemia of infancy. The cell at the right contains a ring, a nuclear particle, and basophilic granules. Leishman's stain (photographs, × 1000).

stain red or reddish purple with Wright's stain and have been thought to be the remains of a nuclear membrane.

Significance of Nucleated Red Corpuscles.—Normally, erythroblasts are present only in the blood of the fetus and of very young infants. In the healthy adult they are confined to the bone-marrow and they appear in the circulating blood only in disease, where their presence denotes an excessive demand made upon the blood-

forming organs to regenerate lost or destroyed red corpuscles. In response to this demand immature and imperfectly formed cells are thrown into the circulation. Their number, therefore, is usually regarded as an indication of the extent to which the bone-marrow reacts rather than of the severity of the disease. A contributing factor may be a "lowered bone-marrow threshold" which allows immature cells to pass into the circulation more readily at some times than at others. Sometimes great numbers of nucleated red corpuscles appear rather suddenly. This is called a **blood crisis,** and the name is rather arbitrarily applied whenever there are present more than five nucleated red cells for every hundred leukocytes.

In general normoblasts appear when blood regeneration takes place in a normal manner, although with excessive activity. They are found in severe symptomatic anemia, leukemia, and pernicious anemia. They are most abundant in myelogenous leukemia. While always present in pernicious anemia, they are often difficult to find. Microblasts have much the same significance as normoblasts, but are less common. Nuclear particles, or Howell-Jolly bodies, are most common in pernicious anemia and have been noted in greatest numbers after splenectomy.

The presence of megaloblasts indicates a change in the type of blood regeneration. This is seen most characteristically in pernicious anemia and the finding of megaloblasts is therefore extremely important in the diagnosis of this disease, although less significance is attached to them than formerly. They are probably present in every case, although often intermittently or in so small numbers as to require a long search; and in well-marked cases they generally exceed normoblasts in number—a ratio which is very rare in other diseases in which they have been found, such as myelogenous leukemia, or malignant growths in the bone-marrow.

2. The Leukocytes.—An estimation of the number or percentage of each variety of leukocyte in the blood is called a differential count. It probably yields more helpful information than any other single procedure in blood examinations.

The **differential count** is best made upon a film stained with Jenner's, Wright's, or a similar stain. Wright's stain is probably most widely used, but differentiates the leukocytes somewhat less satisfactorily than Jenner's. The blood-film need not be quite so thin as is required for study of the red cells, but it must be thin

enough to enable one to identify the leukocytes without difficulty. One should first glance over the preparation to find what the general tinting of the cells may be. Two films stained side by side will often show marked differences in the color reactions of the cells.

To make the differential count, go carefully over the film with an oil-immersion lens, using a mechanical stage if available. Experienced workers often use the lower powers (even the 16-mm., as recommended by Simon) in routine work; but the film must then be mounted, or at least wet with water or oil, since these lenses cannot be used satisfactorily upon dry, unmounted films. Classify each leukocyte seen, and calculate what percentage each variety is of the whole number classified. For accuracy, 500 to 1000 leukocytes must be classified; for approximate results, 300 are sufficient, but it is imperative to count cells in all parts of the smear, since the different varieties may be unevenly distributed. Track of the count may be kept by placing a mark for each leukocyte in its appropriate column, ruled upon paper. Some workers divide a slide-box into compartments with slides, one for each variety of leukocyte, and drop a coffee-bean into the appropriate compartment when a cell is classified. When a convenient number of coffee-beans is used (any multiple of 100) the percentage calculation is simple. Leukocytes which cannot be classified should be placed together in an "unidentified group." In some pathologic conditions, notably leukemia, there may be many of these unidentified cells.

The actual number of each variety in a cubic millimeter of blood is easily calculated from these percentages and the total leukocyte count, and should form part of the record if this is to be complete. An increase in actual number is an *absolute increase;* an increase in percentage only, a *relative increase.* It is evident that an absolute increase of any variety may be accompanied by a relative decrease.

One should make it a rule, when making a differential count, always to attempt to estimate the total leukocyte count from the number of leukocytes seen in a field with the low-power objective. After some practice this can be done with a considerable degree of accurary.

The number of nucleated red corpuscles seen while making the count is generally noted in the record.

It is an unfortunate fact that differential leukocyte counts as ordinarily made on films on slides are often extremely unreliable, owing to irregular distribution of leukocytes, which may be very marked in thin films. For this reason many workers totally condemn the use of slides for differential counting. Undoubtedly, good cover-glass films, made as described on page 268, allow a really accurate differential count, but only when every leukocyte on both covers is classified, which is impracticable in routine work, since it requires classification of 8000 to 30,000 leukocytes. The distribution of leukocytes on the covers is not, however, more uniform than in properly prepared films on slides. In class work it is preferable to use slides, and to require that the films be moderately thick, but not thick enough to render the leukocytes difficult of recognition. A definite number of leukocytes (100 or 200) is then classified in each of three areas extending across the film, one at the beginning, one in the middle, and one at the end, and *reaching to the very edges of the film.* When the count is made in this way the percentage of polymorphonuclear neutrophils (which is taken as the criterion because of the unlikelihood of errors in classification) usually agrees within 2 points with the true percentage as ascertained by classifying every leukocyte on two cover-glass films made from the same puncture.

Proposals have been made from time to time to carry out the differential count in the counting chamber along with the total count by use of diluting fluids which color the leukocytes differentially. None of these has found general favor because of the impossibility of securing good differentiation of all types. The method is most useful when one is interested in the percentage of only one type, as, for example, the eosinophil in suspected trichinosis. As a special diluting fluid for this purpose Stitt uses 1.5 c.c. of neutralized formalin in 98.5 c.c. of 0.5 per cent. glycerol. Just before use this fluid is colored with Giemsa's stain by adding 1 drop of the stain for each 1 c.c. of the fluid. Formalin may be neutralized by adding a few drops of phenolphthalein indicator and then adding very dilute sodium hydroxid to the first appearance of a pink color.

Regarding the nomenclature and classification of some of the leukocytes, particularly those found in pathologic conditions, there is much confusion. In the following pages we shall try to describe these cells with sufficient clearness to facilitate their recognition, and shall, as far as possible, avoid disputed ground, particularly the tangled web of the conflicting theories of histogenesis. The student should thoroughly familiarize himself with the five types

of leukocytes found in normal blood and with at least three—myelocytes, myeloblasts, lymphoblasts—of those which appear in disease.

The following leukocytic percentages represent about the average for normal persons in this country:

	Per cent.
Lymphocytes	25–33
Endothelial leukocytes	2– 6
Polymorphonuclear neutrophils	60–70
Eosinophils	1– 4
Basophils	0.25–0.5

Recent studies indicate that variations among healthy individuals may be greater than has been supposed and that climatic factors or altitude may exert a decided influence. One should, therefore, hesitate to base diagnostic conclusions upon slight variations in the differential count unless one has previously determined the normal for the individual.

(1) **Normal Varieties.**—(*a*) **Lymphocytes** are small mononuclear cells without specific cytoplasmic granules (Frontispiece). They are about the size of a red corpuscle or slightly larger (6–10 μ), although their diameter is influenced to a great degree by the thickness of the film, being greatest in very thin films where the cells are much flattened. The typical lymphocyte consists of a single, sharply defined, deeply staining nucleus, surrounded by a narrow rim of protoplasm. The nucleus is generally round, but is sometimes indented at one side and generally contains one or two dark and very poorly defined nucleoli. Wright's stain gives the nucleus a deep purple color, and the cytoplasm, a robin's-egg blue in typical cells. Larger lymphocytes, 12 to 15 μ in diameter, with paler nuclei and more abundant cytoplasm, are frequently found, especially in the blood of children, and are difficult to distinguish from the endothelial leukocytes. It is believed that the larger forms are young lymphocytes, which become smaller as they grow older. Some workers record the large and small lymphocytes separately. There is no clear line of distinction, but if it seems desirable to separate them, the terms "immature" and "mature" may appropriately be used. In the cytoplasm of many of the larger lymphocytes the Romanowsky stains show a variable number, usually 5 to 10, of rounded, discrete, reddish-purple (azurophilic) granules.

They are larger than the granules of neutrophilic leukocytes and are regarded by some as specific for the lymphocyte.

Lymphocytes are formed in the lymphoid tissues, including that of the bone-marrow. They constitute about 25 to 33 per cent. of all leukocytes, or 1200 to 3000 in each cubic millimeter of blood. They are more abundant in the blood of children, averaging about 60 per cent. in the first year of life and decreasing to about 36 per cent. in the tenth, the immature cells being especially abundant.

The percentage of lymphocytes is usually moderately increased in those conditions which give leukopenia, especially chlorosis, pernicious anemia, and many debilitated conditions. There is a decided absolute and relative increase at the expense of the poly-

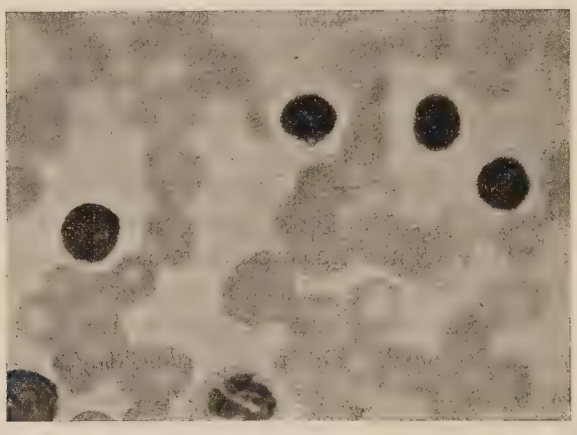


FIG. 138.—Lymphocytosis, case of pertussis (photograph, × 1000) (courtesy of Dr. W. P. Harlow).

morphonuclears at high altitudes, although the extent of this is somewhat uncertain. A similar increase is noted in residents of the tropics and in persons who are much exposed to the sun in temperate zones, as in heliotherapy, where the percentage of lymphocytes seems to increase in proportion to the tanning of the skin. A marked increase, accompanied by an increase in the total leukocyte count, is seen in pertussis (Fig. 138) and lymphatic leukemia. In the former lymphocytes average about 60 per cent. In the latter they sometimes exceed 98 per cent. Exophthalmic goiter commonly gives a marked relative lymphocytosis, while simple goiter does not affect the lymphocytes. In pulmonary tuberculosis a high percentage of lymphocytes or, especially, a progressive increase is a favorable prognostic sign, while a progressive decline

should be looked upon with apprehension. Downing and Allison found a marked increase of lymphocytes in tuberculous patients after induction of artificial pneumothorax.

There is at present a tendency toward greater conservatism in ascribing diagnostic significance to lymphocytosis of moderate degree, that is, of less than 40 per cent., unless the normal for the individual has been previously established. Lymphocytic percentages as low as 15 or as high as 45 are occasionally met with in apparently healthy individuals.

(*b*) **Endotheliocytes (Endothelial Leukocytes, Monocytes)** (Frontispiece and Fig. 139).—Under this head we include the two types which have long been known as large mononuclear and transitional leukocytes. They are merely different forms or ages of the same cell. Although the name "endotheliocytes" is now widely

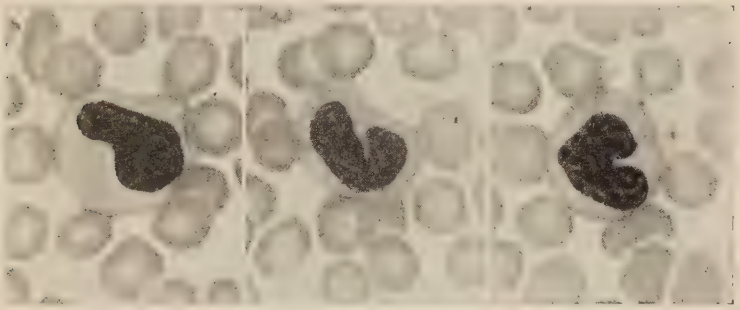


FIG. 139.—Three endothelial leukocytes from normal blood (photographs, × 1000).

used, it has been adopted in the present edition of this book with some hesitancy owing to the uncertainty as to the nature of the cells composing the group, but it undoubtedly serves better than the old names, which have caused much confusion in the past.

The endotheliocyte is the largest cell of normal blood, being generally about two to three times the diameter of a red corpuscle (14–20 μ), although smaller individuals are sometimes encountered. It contains a single nucleus, which is lobulated, deeply indented, or horseshoe-shaped, or, less often, round or oval, and which is commonly located eccentrically.

The zone of protoplasm surrounding the nucleus is relatively wide. With Wright's stain the nucleus is less deeply colored than that of the lymphocyte, while the cytoplasm is very pale blue or colorless, and sometimes appears dusted, uniformly or in patches,

with fine reddish granules which are very much less distinct than the granules of neutrophilic leukocytes. The coarse azurophilic granules characteristic of the immature lymphocytes are not present. The size of the cell, the width of the zone of cytoplasm, and the depth of color of the nucleus are the points to be considered in distinguishing between those endothelial leukocytes which have a round nucleus and lymphocytes, but it must be borne in mind that the thickness of the film has a marked influence upon the apparent size of all leukocytes. They appear larger and paler when flattened out in very thin films. When large forms of the lymphocyte are present the distinction is often difficult or impossible. It is then advisable to class the doubtful cells as lymphocytes. Some workers arbitrarily adopt the size of the polymorphonuclear neutrophil as the dividing line between the two cells.

Comparatively little is known regarding the origin of the endothelial leukocytes, and it is possible that more than one cell is included. Altogether they constitute about 2 to 6 per cent. of the total number of leukocytes; 100 to 600 for each cubic millimeter of blood. Only a few pathologic conditions raise this figure to any marked degree. A distinct increase, to 15 per cent. or even higher, is a feature of the blood in typhoid fever and may be of some value in differential diagnosis. It is also quite constant in malaria, where sometimes many of the cells contain engulfed pigment (Plate VII). Bunting has found it also constant early in Hodgkin's disease and regards it as an extremely important point in diagnosis. Late in the disease it is still evident, but is then overshadowed by an increase of neutrophils. In chronic tetrachlorethane poisoning Minot and Smith have demonstrated a marked and progressive increase of endothelial leukocytes (12–40 per cent.). Other conditions in which an increase is usual are endocarditis lenta, possibly due to proliferation of capillary endothelium (Wollenberg), chronic amebic dysentery, Rocky Mountain spotted-fever, trypanosomiasis, and kala-azar.

(*c*) **Polymorphonuclear Neutrophilic Leukocytes** (Frontispiece).—There is usually no difficulty in recognizing these cells. Their average diameter (about 12 μ) is decidedly less than that of the endothelial leukocytes. The nucleus stains rather deeply, and is very irregular, often assuming shapes comparable to letters of the alphabet, E, Z, S, and so forth (Fig. 140). Frequently there

appear to be several separate nuclei, hence the widely used name, "polynuclear leukocyte." Upon careful inspection, however, delicate nuclear bands connecting the parts can usually be seen. The cytoplasm is relatively abundant, and contains great numbers of fine neutrophilic granules (Fig. 144, A). With Wright's stain the nucleus is purple and the cytoplasmic granules are lilac, while the cytoplasm itself is colorless, or, in the better stained preparations, light pink.

Polymorphonuclear neutrophilic leukocytes are formed in the bone-marrow from neutrophilic myelocytes. Ordinarily they con-

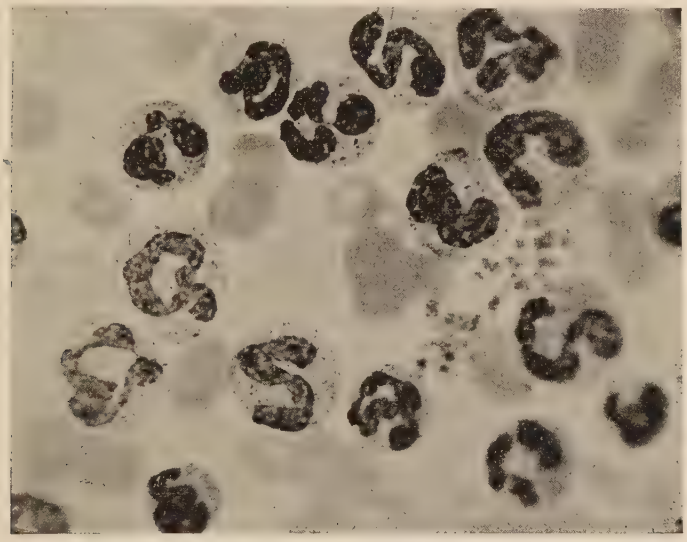


FIG. 140.—Marked polymorphonuclear neutrophilic leukocytosis (photograph, × 1000) (courtesy of Dr. W. P. Harlow).

stitute 60 to 70 per cent. of all the leukocytes: 3000 to 7000 for each cubic millimeter of blood. An occasional normal adult may give a count as low as 40 per cent. or as high as 80 per cent. In children the average runs from about 35 per cent. in the first year to 50 per cent. in the tenth. Any marked increase in their number practically always produces an increase in the total leukocyte count, and has already been discussed under Neutrophilic Leukocytosis (p. 257). The leukocytes of pus, *pus-corpuscles*, belong almost wholly to this variety.

In infectious and inflammatory conditions, notably in pneumonia and appendicitis, a comparison of the percentage of neutro-

philic cells with the total leukocyte count yields more information than a consideration of either alone. In a general way, as was first pointed out by Sondern, the percentage represents the severity of the infection or, more correctly, the *degree of toxic absorption;* while the total count indicates the patient's *power of resistance.* With moderate infection and good resisting powers the leukocyte count and the percentage of neutrophils are increased proportion-

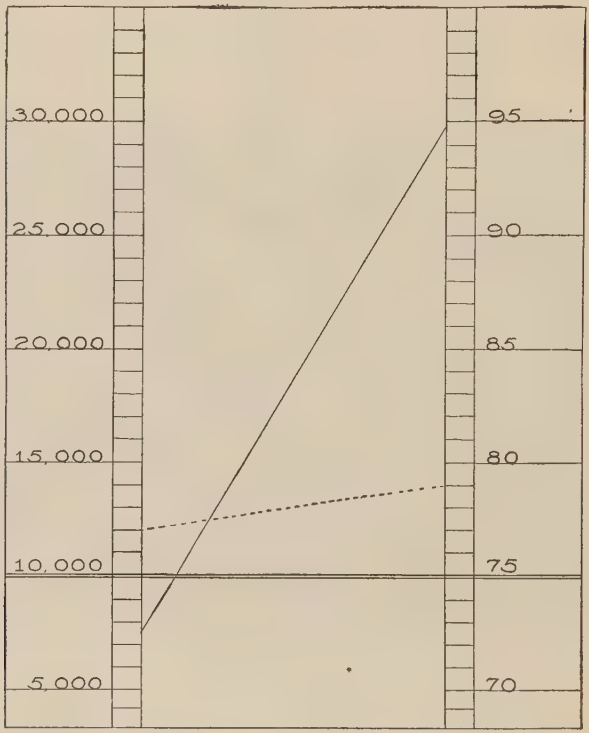


FIG. 141.—Gibson chart with blood-count in 2 cases of appendicitis: Dotted line represents a mild case with prompt recovery; the continuous line, a very virulent streptococcic case with poor resistance, peritonitis, and early death.

ately. When the neutrophilic percentage is increased to a notably greater extent than is the total number of leukocytes, no matter how low the count, either very poor resistance or a very severe infection may be inferred.

Gibson has suggested the use of a chart to express this relationship graphically (Fig. 141). Its arrangement is purely arbitrary, but it will

be found very helpful in interpreting counts. An ascending line from left to right indicates an unfavorable prognosis in proportion as the line approaches the vertical. All fatal cases show a rising line. A descending or horizontal line suggests a very favorable prognosis. Because the chart is somewhat cumbersome in hospital records, when repeated entries must be made, Wilson[1] has devised a formula which expresses the same idea by a numerical index. His formula is as follows: IR = (T − 10) − (P − 70); IR being the "index of resistance," T, the total leukocyte count expressed in thousands, and P, the polymorphonuclear neutrophilic percentage. If, for example, the total count is 12,000 and the neutrophilic percentage is 90, then the formula would give an idex of resistance of − 18: IR = (12 − 10) − (90 − 70) = − 18. Under normal conditions the index will be zero or near it. With low resistance the index is a minus quantity; with disproportionately high resistance it is a plus quantity.

It is a matter of observation that in the absence of acute infectious disease or of inflammation directly in the blood-stream (for example, phlebitis, sigmoid sinusitis, septic endocarditis), a neutrophilic percentage of 85 or more points very strongly to gangrene or pus formation somewhere in the body. On the other hand, excepting in children, where the percentage is normally low, pus is uncommon with less than 80 per cent. of neutrophils.

Exceptions to these rules occur chiefly in moribund cases, in children, and in typhoid and tuberculous infections.

Normally, the cytoplasm of leukocytes stains pale yellow when films are subjected to fumes of iodin and mounted in syrup of levulose. In suppurative and septic conditions the cytoplasm of many of the neutrophils stains diffusely brown, or contains granules which stain reddish brown with iodin. This is called iodophilia. Extracellular iodin-staining granules, which are present normally, are more numerous in iodophilia. Iodophilia was at one time considered a useful sign of suppuration, but is now little valued.

Arneth's Classification of Neutrophils.—Arneth groups the neutrophilic leukocytes into five classes according to the number of lobes which their nuclei possess. The forms which fall into each class and the

[1] Wilson, L. B.: Value of Sondern's Differential Leukocyte Resistance Line in the Diagnosis and Prognosis of Acute Appendicitis: Collected Papers of the Mayo Clinic, 1905–1909, p. 280. Also Walker, O. J.: Index of Body Resistance in Acute Inflammatory Processes, Jour. Amer. Med. Assoc., vol. 72, p. 1453, May 17, 1919.

average normal percentages as given by Arneth are indicated in the following list:

Class 1. One round or indented nucleus; 5 per cent.
Class 2. Two nuclear divisions; 35 per cent.
Class 3. Three nuclear divisions; 41 per cent.
Class 4. Four nuclear divisions; 17 per cent.
Class 5. Five or more nuclear divisions; 2 per cent.

This is really a classification of neutrophils according to their age, the youngest cells being included in Class 1. Among these youngest cells are the myelocytes and metamyelocytes which do not appear in normal blood.

The percentages are fairly constant in the same individual in health, but may show considerable variations in disease, even when the leukocyte count remains unchanged. An increase of the lower classes at the expense of the higher is known as a "shift of the neutrophilic blood-picture to the left." The opposite condition is a "shift to the right." In order to simplify comparison, many workers in this country use an index number obtained by adding the first, second, and one-half of the third classes. The average normal "Arneth index" is, accordingly, about 60. Briggs found variations between 51 and 65 in normal individuals.

The clinical value of an Arneth count is not definitely determined. It appears to have greater usefulness in prognosis than in diagnosis. Most pathologic conditions which produce any change cause a shift to the left, that is, a high index. Among these are acute infectious diseases, pyogenic infections (appendicitis, and so forth), and tuberculosis. In tuberculosis the Arneth count is regarded as having definite prognostic value, the higher the index, the more serious being the outlook.

A low index occurs in pernicious anemia. In a series of twenty-three examinations in twelve cases of pernicious anemia Briggs found an average index of 40.29; lowest, 16.5; highest, 51.25. Eight consecutive cases of severe secondary anemia (malignant disease, syphilis, nephritis, repeated hemorrhages, and so forth) gave an average index of 68.23, only one case (a case of syphilis with index of 39) falling below normal limits.

For the Arneth count thin well-stained blood-films are essential. Wright's stain may be used, but hematoxylin-eosin is better since it brings out the nuclear structure more clearly. Nuclear parts which are joined by more than a thread should be counted as one.

Döhle's Inclusion Bodies.—In 1911 Döhle called attention to the occurrence of certain peculiar bodies within the cytoplasm of the neutrophils in cases of scarlet fever (Fig. 142). Their nature has not been

definitely determined. The typical "inclusion bodies" are about the size of micrococci or a little larger; some of them are pear-shaped, others appear like short rods or like cocci lying in pairs. Smaller, discrete, punctiform granules are sometimes seen, but have not the same significance. It now seems well established that typical inclusion bodies have considerable diagnostic value. They are apparently found in many or even the majority of the neutrophilic leukocytes in every case of scarlet fever early in the disease. Upon the other hand, a few may be found in many cases of diphtheria, pneumonia, and some other infectious diseases, but never in German measles and rarely in measles.

The inclusion bodies can be seen in preparations stained with Wright's stain, but long staining with pyronin-methyl-green is preferable. With the latter stain nuclei are purplish and the bodies bright red.

(*d*) **Eosinophilic Leukocytes, or "Eosinophils"** (Frontispiece.)—The structure of these cells is similar to that of the poly-

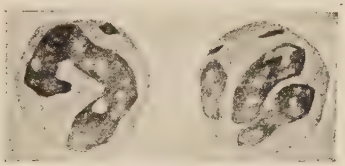


FIG. 142.—Döhle's inclusion bodies in leukocytes. From a case of scarlet fever. Pyronin-methyl-green stain (× 1500) (from a slide prepared by L. W. Hill).

morphonuclear neutrophils, with the striking difference that, instead of fine neutrophilic granules, their cytoplasm contains coarse round or oval granules having a strong affinity for acid stains. They are easily recognized by the size and color of the granules, which stain bright red with stains containing eosin (Fig. 144, B). Their cytoplasm has generally a faint sky-blue tinge, and the nucleus stains somewhat less deeply than that of the polymorphonuclear neutrophil.

Eosinophils are formed in the bone-marrow from eosinophilic myelocytes. Their normal number varies from 50 to 400 for each cubic millimeter of blood, or 1 to 4 per cent. of the leukocytes. An increase is called *eosinophilia*, and is better determined by the actual number than by the percentage.

Marked eosinophilia—above 6 or 7 per cent.—is probably always pathologic. It occurs in a variety of conditions, the most important of which are: infection by animal parasites; bronchial

asthma; myelogenous leukemia; scarlet fever; many skin diseases; anaphylactic conditions; and certain cases of chronic gonorrhea.

(*a*) Eosinophilia may be a symptom of *infection by any of the worms* and from a diagnostic viewpoint this is its most important indication. It is fairly constant in trichiniasis, uncinariasis, filariasis, and echinococcus disease, and reaches its highest figure in the first-named condition where the eosinophils usually range between 10 and 30 per cent. of all the leukocytes, but may go much higher. An unexplained marked eosinophilia, especially if associated with muscular pains, warrants examination of a portion of muscle for *Trichinella spiralis* (p. 512).

(*b*) *True bronchial asthma* commonly gives a marked eosinophilia during and and following the paroxysms. This is helpful in excluding asthma of other origin. Eosinophils also appear in the sputum in large numbers.

(*c*) In *myelogenous leukemia* there is almost invariably an absolute increase of eosinophils, although, owing to the great increase of other leukocytes, the percentage is usually diminished. Dwarf and giant forms are often numerous.

(*d*) *Scarlet fever* is frequently accompanied by eosinophilia, which may help to distinguish it from measles.

(*e*) Eosinophilia has been observed in a large number of *skin diseases*, notably pemphigus, prurigo, psoriasis, and urticaria. It probably depends less upon the variety of the disease than upon its extent.

(*f*) Eosinophilic cells are usually increased to a variable degree tuberculin reactions and subacute and chronic anaphylactic conditions in general, notably in hay-fever.

(*e*) **Basophilic Leukocytes or "Mast-cells"** (Frontispiece).—In general, these resemble polymorphonuclear neutrophils, except that the nucleus is less irregular (usually merely indented or slightly lobulated) and that granules are larger and have a strong affinity for basic stains. They are easily recognized (Figs. 143 and 144, C). Sometimes one sees cells from which most of the granules have disappeared owing to their ready solubility in water, leaving clean-cut openings in the cytoplasm, which then takes a mauve color. With Wright's stain the granules are deep purple, while the nucleus is somewhat paler and is often nearly or quite hidden by the granules, so that its form is difficult to make out.

There is some uncertainty as to the origin of the basophilic leukocytes. Most authorities believe that they originate in the bone-marrow from basophilic myelocytes. They are least numerous of the leukocytes in normal blood, rarely exceeding 0.5 per cent., or

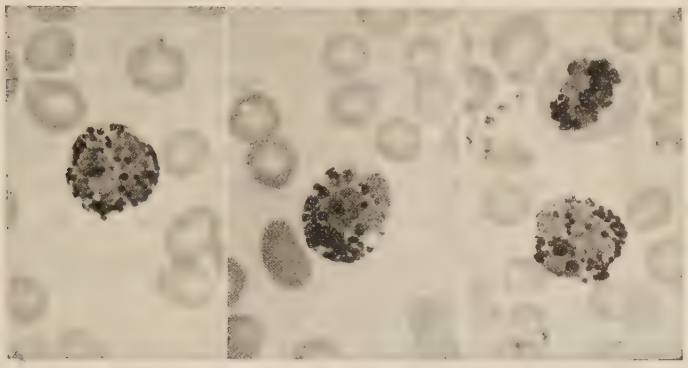
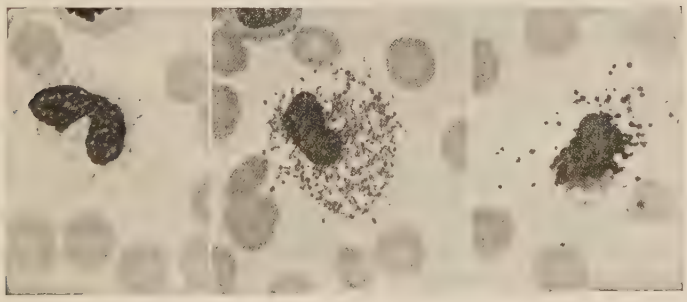


FIG. 143.—Basophilic leukocytes. At the right is also a normoblast undergoing mitosis (photographs, × 1000).

25 to 50 for each cubic millimeter. A notable increase is limited almost exclusively to myelogenous leukemia, where they are sometimes very numerous.

(2) **Abnormal Varieties.**—(*a*) **Myelocytes** (Frontispiece and Fig. 145) are large, mononuclear cells whose cytoplasm is filled

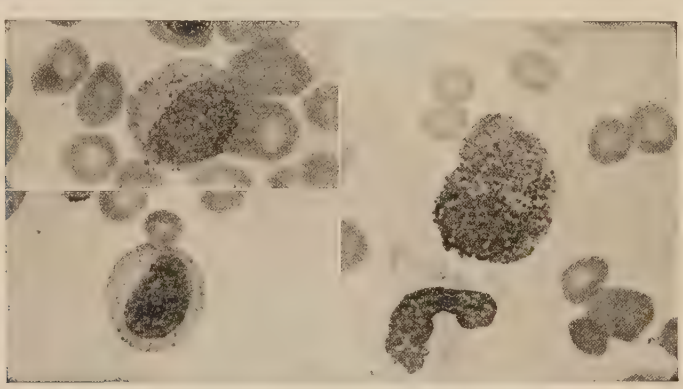


A B C

FIG. 144.—Ruptured leukocytes, showing relative size of granules: A, Neutrophilic; B, eosinophilic; C, basophilic (photographs, × 1000).

with granules. Typically, the nucleus occupies about one-half of the cell, and is round or oval, or is indented, with its convex side in contact with the periphery of the cell. It stains rather feebly and rarely contains nucleoli. The average diameter of this cell (about 15.75 μ) is greater than that of any other leukocyte, but

there is much variation in size among individual cells. Myelocytes are named according to the character of their granules—neutrophilic, eosinophilic, and basophilic myelocytes. These granules are identical with the corresponding granules in the leukocytes just described. They are, however, often less distinct and less sharply differentiated by the various stains than those of the corresponding polymorphonuclear cells. In some the granules are few in number, the cells departing but little from the structure of the parent myeloblast. Such cells may be called "premyelocytes." In young neutrophilic myelocytes there is a tendency to relatively large granules which take a purple color with Wright's stain. These finally give place to true neutrophilic granules. Sometimes only a



A B

FIG. 145.—Myelocytes from blood of myelogenous leukemia: A, Neutrophilic; B, eosinophilic (photographs, × 1000).

portion of the cytoplasm near the nucleus is filled with granules, the periphery, or at least one edge, retaining the smooth basophilic cytoplasm of the parent myeloblast (Frontispiece, Fig. 9). Although the occurrence of two kinds of granules in the same cell is rare, a few basophilic granules are sometimes seen in young eosinophilic myelocytes. The basophilic myelocyte is usually small; and its nucleus is commonly so pale and so obscured by the granules that the cell is not easily distinguished from the mast-cell.

The small neutrophilic cell with a single small, round, deeply-staining nucleus which is sometimes encountered, must not be confused with the myelocyte. Such atypic cells probably result from division of polymorphonuclear neutrophils.

Myelocytes are the bone-marrow cells from which the corresponding granular leukocytes are developed. They, in turn, are derived from certain non-granular cells of the bone-marrow, the myeloblasts. Their presence in the blood in considerable numbers is diagnostic of myelogenous leukemia. The neutrophilic form is the least significant. A few of these may be present in very marked leukocytosis or any severe blood condition, as pernicious anemia. In the anemia of malignant disease they suggest bone-marrow metastasis. Eosinophilic myelocytes are found practically only in myelogenous leukemia, where they are often very numerous. The basophilic variety is less common, and is confined to long-standing, severe myelogenous leukemia.

(*b*) **Myeloblasts.**—These are the parent cells of the myelocytes, from which they differ chiefly in the absence of cytoplasmic granules. They are about the size of myelocytes. Their round or oval nuclei are poor in chromatin, have a finely reticular structure, and contain several rather indistinct nucleoli which are generally pale blue with the usual stain and are outlined by a ring of denser chromatin (Frontispiece and Fig. 146). The cytoplasm, which is generally not abundant, is markedly basophilic, staining pure blue with Wright's stain. In some preparations it is characteristically smooth in texture; in others it is finely reticular.

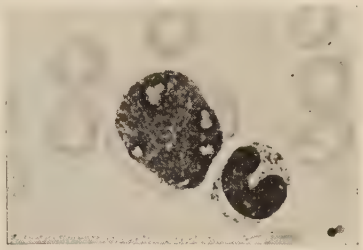


FIG. 146.—A myeloblast and a neutrophilic leukocyte. From a case of myelogenous leukemia. Wright's stain (photograph, × 1000).

Myeloblasts appear in the blood in large numbers in acute myelogenous leukemia and the terminal stages of chronic myelogenous leukemia, when the bone-marrow reverts to the embryonic type. Their number is, therefore, important in prognosis. They may be indistinguishable morphologically from the lymphoblasts of acute lymphatic leukemia, but can usually be distinguished by the peroxidase reaction. In almost all advanced cases of myelogenous leukemia all stages of transition between the myeloblast and myelocyte may be found.

Peroxidase Test.—The technic of Goodpasture, which we have found most satisfactory, is as follows:

1. Prepare dried films on slides or covers in the usual way.

2. Cover the film with a measured amount of Goodpasture's stain and let stand one minute.

3. Add an equal amount of water and let stand three or four minutes.

4. Rinse well in water, dry by blotting or by waving over a low flame, and examine with an oil-immersion objective. By this method nuclei are clear red, cytoplasm and platelets are pale pink, and red corpuscles a buff color. Cells which give the peroxidase reaction—polymorphonuclear neutrophils, eosinophils, myelocytes, myeloblasts, and endothelial leukocytes—contain sharply defined deep blue granules. Such granules are lacking in lymphocytes.

Goodpasture's stain is as follows:

Alcohol	100.00 c.c.
Sodium nitroprussid	0.05 gm.
Benzidin C. P. (Harmer)	0.05 "
Basic fuchsin	0.05 "
Hydrogen peroxid	0.5 c.c.

Dissolve the nitroprussid in 1 or 2 c.c. of water, mix with the alcohol, and then add the other ingredients. In order to secure satisfactory staining of the nuclei we have found it necessary to double the amount of fuchsin, owing probably to a difference in strength of dye.

Goodpasture's stain remains good only a few days. Beacom has found that it will give satisfactory results for eight months if made up without the peroxid. A freshly made 1 : 200 dilution of hydrogen peroxid is then used in place of water for diluting the stain on the slide. This solution is made with sufficient accuracy by adding 2 drops of hydrogen peroxid to 15 c.c. distilled water.

(*c*) **Lymphoblasts.**—In acute lymphatic leukemia there appears in the blood a high percentage of very young cells of the lymphocytic series. To these the name lymphoblasts is given. They are identical with the large cells of the germinal centers of the lymph-nodes. In many cases at least they are indistinguishable from the large or immature lymphocytes previously described as occasionally occurring in the blood of normal adults and frequently in that of children. Azure granules are sometimes seen in the cytoplasm, and the nucleus generally shows one or two nucleoli (Frontispiece). At times the nucleus is curiously lobulated and the name "Rieder's cell" is then applied. Figure 1b of Plate VIII shows several Rieder's cells.

Lymphoblasts are sometimes morphologically indistinguishable from myeloblasts, but do not give the peroxidase reaction.

(*d*) **Türck's Irritation Leukocytes.**—These are large, mononuclear, non-granular cells with dense, opaque, strongly basophilic cytoplasm which often contains vacuoles (Frontispiece). With Wright's and similar stains the cytoplasm stains almost as intensely as the nucleus, although of a different color, being deep blue, while the nucleus is deep purplish red. The nuclear chromatin shows no tendency to radial arrangement. Nucleoli are rarely or never present.

The nature of the irritation leukocytes is not definitely known and at present no diagnostic importance can be ascribed to them. Some believe them to be pathologic myeloblasts. Considerable numbers have been found in the blood in conditions associated with irritation of the bone-marrow, notably primary and secondary anemia, leukemia, and malaria, and in the leukocytosis of pneumonia.

(*e*) **Plasma Cells.**—Morphologically very similar to Türck's irritation leukocyte, the plasma cell is said to be distinguished by the presence of one or two nucleoli in its nucleus and especially by a tendency toward radial or "wheel-like" arrangment of its chromatin (Frontispiece).

Plasma-cells are apparently an altered form of the lymphocyte, the same cell which appears so frequently at the site of chronic inflammation of certain types. They are extremely rare in the circulating blood and have no diagnostic significance.

(*f*) **Degenerated Forms.**—These are frequently met, but have no significance unless present in large numbers. They include (*a*) vacuolated leukocytes and (*b*) bare nuclei from ruptured cells. The former are found most frequently in toxemias and leukemia. A few of the latter are present in every blood-smear, but are especially abundant in leukemia (Fig. 147). They vary from fairly well-preserved nuclei without cytoplasm to mere strands of palely stained nuclear substance arranged in a coarse network—the so-called "basket-cells" (Frontispiece and Fig. 148).

Occasionally in lymphatic leukemia frayed-out nuclei without cytoplasm exceed the usual lymphocytes in number. In such cases some writers infer involvement of the bone-marrow, holding that the naked nuclei represent very fragile bone-marrow cells which

have gone to pieces in the circulation. In many cases, at least, it seems more likely that such nuclei only represent fragile lymphocytes which have been broken in making the smear.

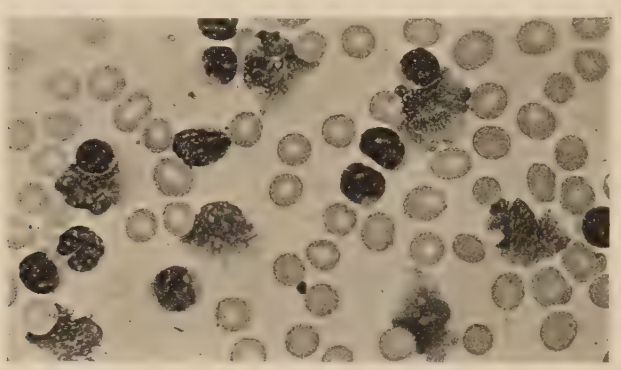


FIG. 147.—Blood in chronic lymphatic leukemia, showing many ruptured lymphocytes (photograph, × 750).

(*g*) **Atypic Forms.**—Leukocytes which do not fit in with the above classification are not infrequently met, especially in high-grade leukocytosis, pernicious anemia, and leukemia. They are always more abundant in childhood. The nature of many of

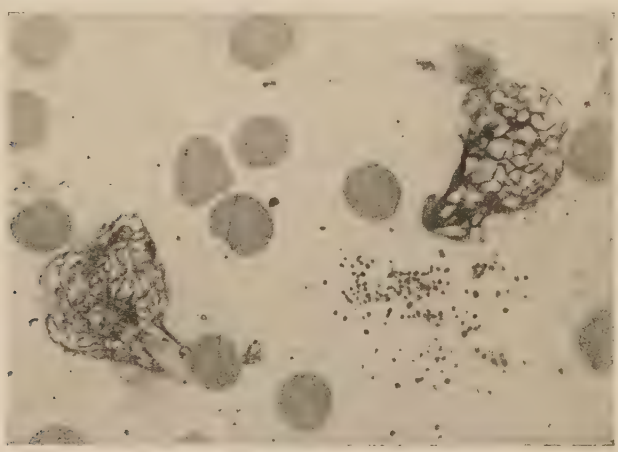


FIG. 148.—Two remnants of degenerated nuclei in a blood film. They are often called "basket cells" (photograph, × 1000).

them is not clear, and their number is usually so small that they may be classed as "undetermined" in making a differential count.

3. Blood-platelets.—These are not colored by hematoxylin and eosin. With Wright's and similar stains they appear as spheric

or ovoid, reddish to violet, granular bodies, 2 to 4 μ in diameter. Occasionally a platelet as large as a red corpuscle is seen. When well stained a delicate pale blue, hyaline, ground substance can be distinguished. In ordinary blood-smears they are usually clumped in masses. A single platelet lying upon a red corpuscle may easily be mistaken for a malarial parasite, while unusually large and oval platelets are occasionally mistaken for estivo-autumnal crescents (Frontispiece and Fig. 149).

Blood-platelets are being much studied at present, but, aside from the facts mentioned under their enumeration (p. 264), little of clinical value has been learned. They have been variously regarded as very young red corpuscles (the "hematoblasts" of Hayem), as disintegration products of leukocytes, as remnants of

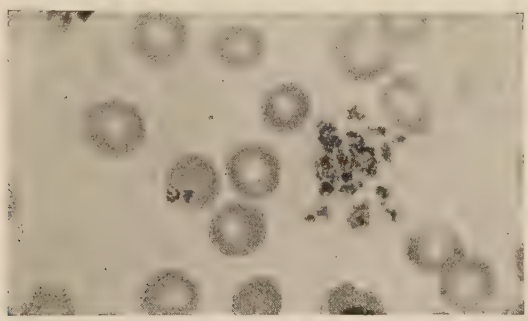


FIG. 149.—A cluster of blood-platelets and two platelets lying upon a red cell and simulating malarial parasites (photograph, × 1000).

extruded nuclei of erythrocytes, and as independent nucleated bodies. At present the accepted explanation of their origin is that of J. H. Wright, who regards them as detached portions of the cytoplasm of certain giant-cells (megakaryocytes) of the bone-marrow. The giant-cells push cytoplasmic processes into the capillaries, and these break off and appear in the circulating blood as platelets.

The megakaryocytes themselves occasionally get into the blood-stream, particularly in conditions in which the bone-marrow is under intense strain as in myelogenous leukemia. Most of them are too large to pass the capillaries and are arrested in the lungs, but the smaller cells may pass the lungs and appear in the peripheral circulation. They are rarely numerous.[1]

[1] For a description of these cells as they appear in blood-films and an excellent colored plate see Minot, G. R.: Megakaryocytes in the Peripheral Circulation, Jour. Exp. Med., vol. 36, p. 1, July, 1922.

X. BLOOD PARASITES

A. Bacteria

Bacteriologic study of the blood is useful in many conditions, but, in general, the somewhat elaborate technic involved in isolating and identifying the bacteria limits its use to the trained laboratory man. As applied to the diagnosis of typhoid fever, however, the technic of blood-cultures is so simple, because of the large number of bacilli usually present early in the disease and because of the ease with which they can be identified, that it can be carried through by anyone who is competent to do the simplest cultural work.

Typhoid bacilli can be detected in the blood in practically every case of typhoid fever in the first week of the disease; in about 80 to 85 per cent. of cases in the second week; and in decreasing percentages in the later weeks. The blood-culture, therefore, offers the most certain means of early diagnosis. It is in a sense complementary to the Widal reaction, the former decreasing and the latter increasing in reliability as the disease progresses. The blood-culture gives best results before the Widal appears, as one would expect from the fact that the Widal test depends upon the presence of antibodies which are inimical to the bacilli. The two methods together will establish the diagnosis in practically every case at any stage. Bacilli disappear from the blood in convalescence and reappear in a relapse.

In general, blood-cultures should be undertaken in all cases of long-continued fever of unknown etiology. Cultures may be more successful if made at a time when the temperature is rising than when it is falling. In septicemia and malignant endocarditis cultures are nearly always positive, the organisms most frequently found being streptococci, staphylococci, and pneumococci. Streptococci, usually *Streptococcus viridans*, or more rarely, staphylococci, can be recovered in the great majority of cases of subacute endocarditis if repeated cultures be made. Among acute infectious diseases pneumonia and acute epidemic meningitis show bacteriemia in about half of the cases. Blood-cultures are almost always negative in otitis media but usually become positive (showing streptococcus as a rule) when the inflammation extends to the lateral sinus or meninges.

Method for Blood-cultures.—*Apparatus.*—It is necessary to have ready a sterile syringe and one or more flasks or tubes of citrate solution or of fluid culture-media.

A high-grade all-glass syringe is best. The syringe and needle may be sterilized by boiling them for ten minutes, or by wrapping in a towel and sterilizing in the autoclave. When it is desired to keep a syringe on hand ready for use it is placed with needle attached, in a large cotton-plugged test-tube, in the bottom of which stands a short section of glass tubing into which the needle is inserted to protect its point; the top of the tube is covered with several layers of gauze which should be folded closely about its neck and tied with a string; and finally the tube and syringe are sterilized in the hot-air oven at 150° C. for one hour. In some laboratories it may be found more satisfactory to keep the syringes and needles on hand simply wrapped in towels and sterilized in the hot-air oven.

A number of small cotton-plugged flasks or large tubes are charged with 5 to 10 c.c. of citrate solution (2 gm. sodium citrate, 0.6 gm. sodium chlorid, 100 c.c. distilled water), sterilized in the autoclave, and kept on hand ready for use. Before sterilization the tops of the flasks or tubes are covered with moderately heavy paper, which is folded neatly about the necks and held in place with rubber bands. Instead of citrate solution some prefer citrated broth or, in certain cases, a fluid medium without citrate, as dextrose-brain broth.

In private practice the use of the Keidel vacuum tube charged with citrated broth is very satisfactory (p. 307).

Technic.—(*a*) Secure 5 to 20 c.c. of blood from a vein in a sterile syringe under strictly aseptic conditions (p. 222).

(*b*) Immediately remove the needle, place the blood in the flasks or tubes of citrate solution described, adding about as much blood as there is solution in the container, and mix by gently shaking. The citrate prevents clotting and facilitates handling in the laboratory later.

(*c*) At the laboratory mix the citrated blood with appropriate media and place in the incubator. Great care must be exercised to avoid contamination of media when the cotton stoppers are removed or replaced. There is less danger of contamination if the blood is transferred by means of a sterile cotton-plugged pipet than if it is poured from flasks or tubes. A portion of the citrated blood is mixed with nutrient agar, which has been melted and cooled to 45° C., and is at once poured into Petri dishes. The usual proportion is about 1 part of citrated blood to 3 or 4 of agar. The remainder is distributed among flasks or tubes of fluid media, usually in the proportion of 1 c.c. of blood to 20 c.c. or more of medium.

(*d*) Examine the cultures daily. Do not discard any as negative for ten days or two weeks. Growth in fluid media containing an indicator is evidenced by change in color of the medium. Staphylococci in pyemia usually grow readily in the first twenty-four hours, and the colonies are deep in the plates as well as on the surface. Growth of a few surface colonies, only, commonly means contamination. On the other hand, streptococci and pneumococci do not appear for forty-eight hours or longer. Zones of hemolysis or of green-pigment production about the colonies of the various types of streptococci or pneumococci are readily noted on the plates.

Instead of using citrate solution as above described, it may be desirable in some cases to place all or part of the blood directly into fluid culture media at the bedside, but a relatively large volume of the medium must then be used, usually no less than 30 c.c. for 1 c.c. of blood.

Culture Media.—The choice of media depends on the organisms expected. The nutrient-agar plates described are generally prepared routinely. A medium containing bile is preferred for bacilli of the typhoid-paratyphoid group. For streptococci and pneumococci and for general purposes, a good medium is nutrient broth to which one-fourth its volume of sterile ascitic fluid has been added. A still better medium is dextrose brain-broth (p. 645). Streptococci and pneumococci grow well in this, especially in the deeper portion. Growth is indicated by a pink color due to formation of slight acidity, as well as by the usual change in turbidity.

Enumeration of Bacteria in Blood.—When it is desired to count the bacteria, a definite amount of blood from a vein, measured as accurately as can be done with a graduated syringe, is mixed at the bedside with a measured amount of the citrate solution described above. At the laboratory definite amounts of this citrated blood are added to tubes of melted nutrient agar by means of a sterile graduated pipet and poured into Petri dishes. When colonies appear, the number of colonies for each plate is counted and the number of bacteria for each cubic centimeter of blood can then be calculated.

Blood-cultures for Typhoid Bacilli.—These are mentioned separately because they offer the most valuable means of early diagnosis of typhoid and paratyphoid fevers, and are so simple, owing to the large number of bacilli usually present in the blood in the first week or two of the disease and to the rapidity with which they grow, as to require little bacteriologic experience or equipment. The blood is obtained from a vein as described and placed at once into a small flask or large tube containing three or four times its volume of ox-bile medium (p. 653) or nutrient broth with 2 per cent. of sodium citrate. Both media prevent

coagulation of the blood, but the bile medium is preferable in private practice because it is very easily prepared, and because it favors the growth of the typhoid group of bacilli while it retards that of other bacteria such as contaminating skin cocci. In every case a small amount of the blood should be reserved for the Widal test.

Cultures are placed in the incubator as soon as possible after the blood is added, and at the end of twelve and twenty-four hours are examined for motile bacilli by the hanging-drop method. If none are found transfers are made to slants of nutrient agar, or better, litmus-lactose agar, and incubated for twelve or twenty-four hours longer. On litmus-lactose agar colonies of contaminating staphylococci are pink. If Gram-negative bacilli are found, they are presumably typhoid or paratyphoid bacilli. Further study is, however, necessary to identify them with certainty, and appropriate media (p. 670) should be inoculated from the growth, or if there be contamination, from isolated colonies obtained as described on page 680. The agglutination test (p. 578) should be used if agglutinating serum is at hand, but it must be remembered that freshly isolated bacteria do not agglutinate well.

Blood-cultures in Keidel Tubes.—A satisfactory method of making blood-cultures for typhoid bacilli and other organisms is the use of Keidel vacuum tubes (Fig. 98). This is very convenient in private practice, and in the small laboratory where little cultural work is done routinely, as it reduces labor and also reduces the likelihood of contamination in handling the blood at the bedside. Tubes charged with ox bile, citrated broth, or other media may be obtained from the manufacturers.[1] After the skin over the vein has been cleaned and the tourniquet applied in the usual way, the cap covering the sterile needle attached to the tube is removed, and the needle is inserted into the vein. The tip of the glass tube is broken through the rubber connection either with the fingers or an artery forceps. Blood will enter because of the vacuum in the tube. Incubation and the other bacteriologic procedures are then carried out.

B. Animal Parasites

Of the animal parasites which have been found in the blood, five are interesting clinically: the "spirochæte" of relapsing fever; trypanosomes; malarial parasites; filarial larvæ, and the larvæ of *Trichinella spiralis*.

1. Borrelia recurrentis is described on page 459.

[1] Hynson, Wescott, and Dunning, Baltimore, Maryland. These tubes may also be obtained from other supply houses.

2. Trypanosoma gambiense.—Various trypanosomes are common in the blood of fishes, amphibians, birds, and mammals (Fig. 191). They live in the blood-plasma and do not attack the corpuscles. In some animals they are apparently harmless; in others they are an important cause of disease. They are discussed more fully on page 463.

The best known trypanosome of human blood, *Trypanosoma gambiense* (Plate VI), is an actively motile, spindle-shaped organism, from two to four times the diameter of a red corpuscle in length, with an undulating membrane which terminates at the anterior end in a long flagellum. It can be seen in stained films with medium-power objectives, but is best studied with an oil-immersion lens. It will be necessary to search many slides. The concentration method described for the larvæ of *Trichinella* (p. 320) may be used, or 10 c.c. of citrated blood may be thoroughly centrifugalized and the trypanosomes sought in the milky leukocytic layer above the solidly packed red corpuscles. Microscopic examination of fluid aspirated from a lymph-node (p. 463) is perhaps the best means of diagnosis. Human trypanosomiasis is common in Africa. As a rule, it is a very chronic disease. "Sleeping sickness" is a late stage when the organisms have invaded the cerebrospinal fluid. Infection is carried by the tsetse-fly, *Glossina palpalis*, in which the trypanosome passes a stage of its development. Further details are given on page 463.

3. The Malarial Parasites.—These protozoa belong to the Sporozoa (p. 470), order Hemosporidia, and family Plasmodiæ, the members of which are parasites in the blood of a great variety of vertebrates. Three species, belonging to the genus *Plasmodium*, are associated with malarial fever in man: *Plasmodium vivax*, *P. malariæ*, and *P. falciparum*, the parasites respectively of the tertian, quartan, and estivo-autumnal types of malaria. The life-histories of the three are so similar that they may well be described together.

(1) **Life-histories.**—There are two cycles of development: one, the *asexual*, in the blood of man, and the other, the *sexual*, in the intestinal tract of female mosquitos of certain species of the genus *Anopheles*.

(*a*) *Asexual Cycle.*—The young organism enters the blood through the bite of the mosquito. It makes its way into a red

PLATE VI

PROTOZOAL PARASITES OF THE BLOOD.
(Photographs, × 1000; 1 mm. = 1 μ).

FIG. 1.—Trypanosoma gambiense.

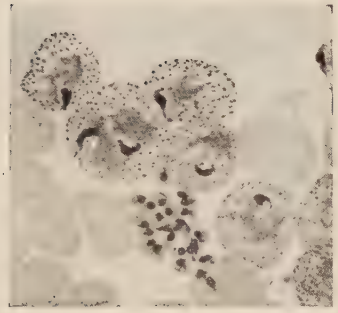


FIG. 2.—Half-grown tertian malarial parasites in stippled cells and a group of spores from a freshly ruptured segmenter. From a slide of double tertian malaria concentrated by F. M. Johns.

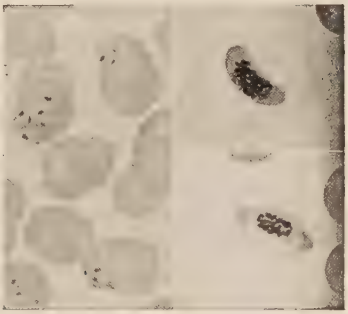


FIG. 3.—Estivo-autumnal malarial parasites, small ring forms and crescents.

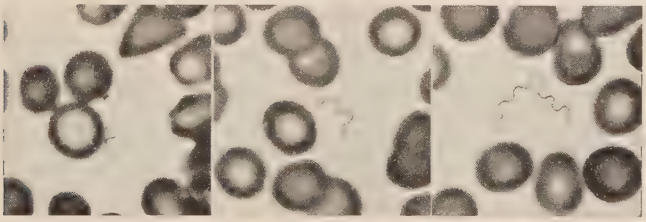


FIG. 4.—Spirochetes in the blood of a case of relapsing fever originating in Colorado. Reported by Dr. C. N. Meader.

corpuscle,[1] where it appears as a small, pale, "hyaline" body, which generally assumes the form of a ring. Later it loses its ring-like shape, increases in size, and exhibits ameboid movements. Within about eight hours dark brown granules, derived from the hemoglobin of the corpuscle, make their appearance within it. When it has reached its full size, almost filling the corpuscle and causing it to enlarge, in the case of the tertian parasite, smaller in the others, the pigment granules gather at the center or at one side; the organism divides into a number of small hyaline bodies, the spores or *merozoites*; and the red corpuscle bursts, setting spores and pigment free in the blood-plasma. This is called segmentation. It coincides with, and by liberation of toxins causes the paroxysm of the disease. A considerable number of the spores are destroyed by leukocytes or other agencies; the remainder enter other corpuscles and repeat the cycle. Many of the pigment granules are taken up by leukocytes. In estivo-autumnal fever segmentation occurs almost exclusively in the capillaries of the internal organs and the segmenting and larger pigmented forms are seldom seen in the peripheral blood. This accumulation of parasites in the internal organs explains certain types of pernicious estivo-autumnal malaria, for example, the comatose type, when the parasites accumulate in the capillaries of the brain. The other malarial parasites show a similar tendency, but it is much less marked than in the case of the estivo-autumnal parasite.

The asexual cycle of the tertian organism occupies forty-eight hours; of the quartan, seventy-two hours; of the estivo-autumnal, an indefinite time—usually twenty-four to forty-eight hours.

The parasites are thus present in the blood in great groups or broods, all the individuals of which reach maturity and segment at approximately the same time. This explains the regular recurrence of the paroxysms at intervals corresponding to the time occupied by the asexual cycle of the parasite. Not infrequently there is multiple infection, one group reaching maturity while the others are still young; but the presence of two groups which segment upon

[1] In this section the malarial parasite is described, in accordance with the usual teaching, as living within the parasitized red corpuscle. The work of Mary Rowley-Lawson, however, tends to show that the parasite is extracellular throughout its whole existence; that it attaches itself to the external surface of the red corpuscle, but does not enter it; and that it migrates from corpuscle to corpuscle between paroxysms, destroying each cell before it abandons it.

the same day is extremely rare. Fevers of longer intervals—six, eight, ten days—are probably due to the ability of the body, sometimes of itself, sometimes by aid of quinin, to resist the parasites, so that numbers sufficient to cause a paroxysm do not accumulate in the blood until after several repetitions of the asexual cycle. In estivo-autumnal fever the regular grouping, while usually present at first, is soon lost, thus causing "irregular malaria."

Bass has succeeded in cultivating the malarial parasite outside of the body.

(*b*) *Sexual Cycle.*—Besides the ameboid individuals which pass through the asexual cycle, there are present with them in the blood many individuals with sexual properties. These are called *gametocytes;* the males, *microgametocytes;* the females, *macrogametocytes.* Like the asexual forms they start as young parasites liberated from the sporulating parent. However, they grow more slowly and, when they reach the adult size, do not undergo segmentation, but remain inactive in the blood until taken up by a mosquito. Many of them are apparently extracellular, but stained preparations usually show them to be surrounded by the remains of a corpuscle. In tertian and quartan malaria they resemble the asexual individuals until a variable time after the blood leaves the body, when the male gametocyte sends out one or more flagella. In estivo-autumnal malaria the gametocytes take distinctive ovoid and crescentic forms, and are not difficult to recognize. These sexual forms are very resistant to quinin, and often persist in the blood long after the ameboid forms have been destroyed. Under ordinary conditions they are incapable of continuing the disease until they have passed through the cycle in the mosquito, but it seems probable that under certain unusual conditions the female gametocyte may, without fertilization, undergo further development and sporulate, thus starting an entirely new asexual cycle.

When a malarious person is bitten by a mosquito, the gametocytes are taken with the blood into its stomach. Here the male sends out one or more flagella known as microgametes. These break off and seek out the females, which have undergone a process of maturation and are now known as macrogametes, whom they fertilize much as the sperm fertilizes the ovum. The fertilized female is then a *zygote;* it becomes elongated and actively motile and finally penetrates the intestine and encysts on its outer wall. This

"oöcyst" grows enormously and projects into the body cavity of the mosquito as a conspicuous knob, easily seen in dissected mosquitos.

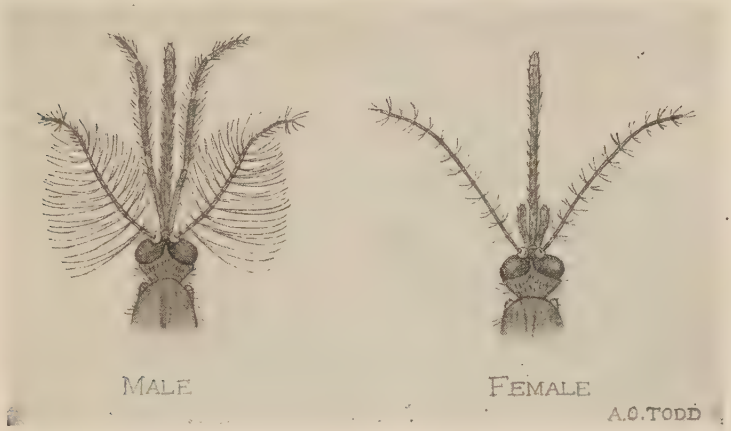


FIG. 150.—Heads of *Culex* (*Culex pipiens*) showing the straight proboscis, the jointed palpi, and external to these the hairy antennæ. The male is distinguished from the female by the longer hairs on the antennæ. Note that the palpi of the male are about as long as the proboscis, while those of the female are very much shorter (compare with Fig. 151).

with very low magnification. After about two weeks the oöcyst ruptures, liberating many minute rods, or *sporozoïtes*, which have

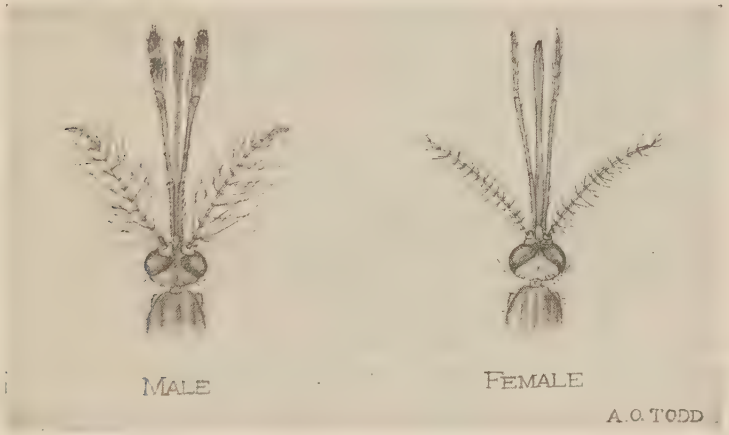


FIG. 151.—Heads of *Anopheles* (*Anopheles maculipennis*). The sexes are distinguished by the antennæ as noted under Fig. 150. The palpi of *Anopheles* are nearly the same length as the proboscis in both sexes.

formed within it. These migrate to the salivary glands, and are carried into the blood of the person whom the mosquito bites. Here they enter red corpuscles as young malarial parasites, and the

majority pass through the asexual cycle just described. Definite symptoms of malaria do not, however, appear until the parasites have multiplied to a sufficient number—usually ten or twelve days in acute cases. Ross estimated that ordinarily about 150,000,000 parasites must be present in the body before symptoms are produced.

The sexual cycle can take place only within the body of the female of certain mosquitos belonging to the genus *Anopheles*. The male does not bite. Absence of these mosquitos from certain

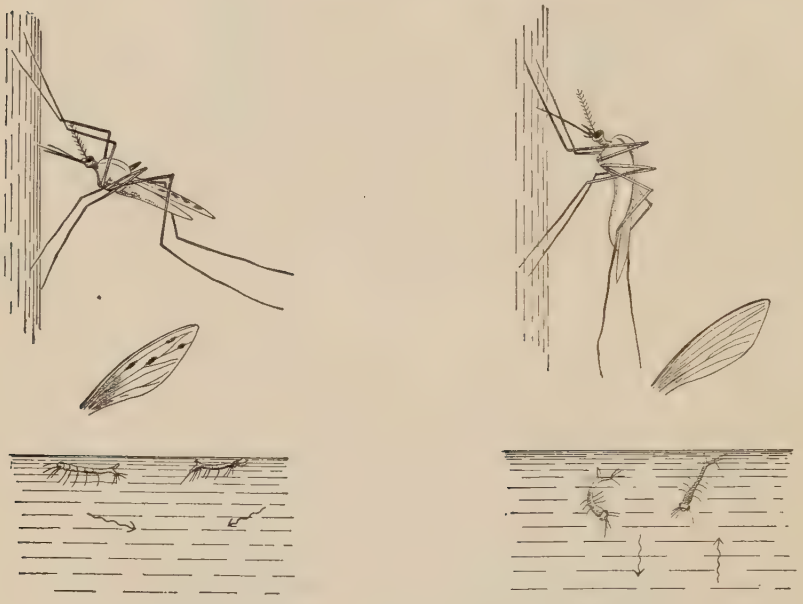


FIG. 152.—Showing, on the left, *Anopheles* in resting position, its dappled wing, and the position of its larvæ in water; on the right, *Culex* in resting position, its plain wing, and the position of its larvæ in water. The arrows indicate the directions taken by the larvæ when the water is disturbed (Abbott).

districts explains the absence of malaria. *Anopheles* is distinguished from other common mosquitos, particularly the widely distributed genus, *Culex*, by the relative lengths of proboscis and palpi (Figs. 150 and 151), which can be seen with a hand-lens, by its attitude when resting, and by the dappled wing which characterizes most species of *Anopheles* (Fig. 152).

Anopheles is strictly nocturnal in its habits; it usually flies low, and rarely travels more than a few hundred yards from its breeding-place, although it may be carried by winds. These facts explain

certain peculiarities in malarial infection; thus, infection occurs practically only at night; it is most common near stagnant water, especially upon the side toward which the prevailing winds blow; and the danger is greater when persons sleep upon or near the ground than in upper stories of buildings. Most of the mosquitos die in the fall, but individuals frequently hibernate in warmed houses, and may bite during the winter. A mosquito becomes dangerous in eight to fourteen days after it bites a malarious person, and remains so throughout its life.

One other mosquito which is very important from a medical point of view may be mentioned at this place. This is *Aëdes ægypti* (*Stegomyia fasciata*) which is the carrier of yellow fever and is probably concerned in the transmission of dengue. It is a small mosquito and is distinguished by two curved and two straight silver white markings, suggesting a lyre, on the back of the thorax, and by bands of similar color around the abdomen and legs (Fig. 153). This and nearly all other members of the genus *Aëdes* have palpi long in the male and short in the female, as in *Culex*.

FIG. 153.—*Aëdes ægypti* (*Stegomyia fasciata*), the yellow fever mosquito. Female. Note the lyre-like markings on the back of the thorax (Boyce, after Newstead).

(2) **Detection.**—Search for the malarial parasite may be made in either fresh blood or stained films. If possible, the blood should be obtained a few hours before the chill—not during it or within a few hours afterward, since at that time (in single infections) only the very young, unpigmented forms are present, and these are the most difficult to find and recognize. Sometimes many parasites are found in a microscopic field; sometimes, especially in estivo-autumnal infection, owing to accumulation in internal organs, careful search is required to find any, despite severe symptoms. In

ordinary acute malaria, however, the parasites can usually be found within five to twenty minutes. Only in chronic cases and in "malaria carriers" is it necessary to resort to concentration methods. Quinin causes them rapidly to disappear from the peripheral blood, and few or none may be found after its administration. In the absence of organisms the presence of pigment granules within leukocytes—especially the endotheliocytes—may be taken as definite evidence of malaria. Pigmented leukocytes (Plate VII) are most numerous after a paroxysm and in chronic malaria.

(*a*) *In Fresh Unstained Blood.*—Obtain a small drop of blood from the finger or lobe of the ear. Touch the center of a coverglass to the top of the drop and quickly place it, blood side down, upon a slide. If the slide and cover be perfectly clean and the drop not too large, the blood will spread out so as to present only one layer of corpuscles. Search with an oil-immersion objective, using very subdued light. The preparation may be kept for many hours if ringed with vaselin or melted paraffin.

The young organisms appear as small, round, ring-like or irregular, colorless bodies within red corpuscles. The light spots caused by crenation and other changes in the corpuscles are frequently mistaken for them, but are generally more refractive or have more sharply defined edges. The older forms are larger colorless bodies containing granules of brown pigment. In the case of the tertian parasite, these granules have active vibratory motion, which renders them conspicuous; and as the parasite itself is very pale, one may see only a large pale corpuscle in which fine pigment granules are dancing. Segmenting organisms, when typic, appear as rosets, often compared to daisies, the petals of which represent the segments, while the central brown portion represents the pigment. Tertian segmenting forms are less frequently typic than quartan. Flagellated forms are not seen until ten to twenty minutes after the blood has left the vessels. As Cabot suggests, one should, while searching, keep a sharp lookout for unusually large or pale corpuscles, and for anything which is brown or black or in motion.

The table on page 315 contrasts the distinguishing characteristics of the three species as seen in fresh unstained blood.

(*b*) *In Stained Films.*—Recognition of the parasite, especially the young forms, is much easier in films stained by Wright's or

SPECIES OF THE MALARIAL PARASITE

Tertian.	Quartan.	Estivo-autumnal.
Asexual cycle, forty-eight hours.	Seventy-two hours.	Usually twenty-four to forty-eight hours.
Substance pale, transparent, comparable to hyaline tube-cast.	Highly refractive, comparable to waxy tube-cast.	Highly refractive.
Outline indistinct.	Distinct.	Distinct.
Ameboid motion active.	Sluggish.	Active.
Mature asexual form large; fills and often distends corpuscle.	Smaller.	Young forms, only, in peripheral blood.
Pigment-granules fine, brown, scattered throughout. Very active dancing motion.	Much coarser, darker in color, peripherally arranged. Motion slight.	Very few, minute, inactive. Distinctly pigmented forms seldom seen.
Segmenting body rarely assumes typical "daisy" form. 15 to 20 segments.	Usually typical "daisy." 6 to 12 segments.	Very rarely seen in peripheral blood.
Gametocytes resemble asexual forms.	Same as Tertian.	Appear in blood as distinctive ovoids and crescents.
Red corpuscles pale and swollen.	Generally darker than normal.	Dark, often bronzed.

some similar stain than in fresh blood. The films must be thin and well stained. It is useless to search preparations in which the nuclei of leukocytes are not strongly colored.

In films which are properly stained with Wright's or Giemsa's stain malarial parasites appear as follows:

The **young parasites** (Plate VII, Figs. 1, 4, and 5, *a*) are small, round, ring-like or irregular, sky-blue bodies, each with a very small, sharply defined, purplish-red chromatin mass. Many structures—deposits of stain, dirt, blood-platelets lying upon red cells (Frontispiece and Fig. 149), and so forth—may simulate them, but should not deceive one who looks carefully for both the blue cytoplasm and the purplish-red chromatin. A platelet upon a red corpuscle is surrounded by a colorless zone rather than by a distinct blue body and there is no compact chromatin mass. The highly refractile colorless spots sometimes produced in red corpuscles by slow drying of the film are often mistaken for parasites by beginners. When a little out of focus these may show much

color, owing to chromatic aberration of the objective. As quartan parasites grow a little older they tend to assume a straight or slightly curved, band-like form which is fairly characteristic of this species. Young estivo-autumnal parasites commonly take the form of small, delicate, blue rings, each with one or two small purplish-red chromatin bodies upon its circumference. Their recognition is important because they may be the only form found in a given case. When young tertian parasites assume this form the ring is usually larger and thicker. Usually, it is the dot-like chromatin body which first attracts one's attention to the parasitized cell. In tertian malaria the fact that cells which harbor the parasites are somewhat larger and paler than their fellows is also helpful in attracting one's attention while searching. This may be evident as early as eight hours after the chill. No such enlargement of the red cells is noted in other forms of malaria.

Older tertian and **quartan parasites** (Plate VII, Figs. 4 and 5, *b*) show larger, irregularly shaped, sky-blue bodies with more abundant, paler, and more reticular or granular chromatin, and contain brown granules of pigment, which, however, are less evident than in the living parasite. The chromatin usually lies in a colorless area or "achromatic zone," and is sometimes so pale as to be difficult to see clearly. Not infrequently it appears to lie entirely outside of the cytoplasm. The pigment of the adult tertian parasite is usually fine and scattered uniformly through the cytoplasm. That of the quartan is coarser and more peripherally arranged. The quartan parasite is smaller than the tertian and does not enlarge the red corpuscle, which is often even smaller and more darkly colored than the surrounding unparasitized corpuscles. Very characteristic is its tendency to take the form of a broad band extending across the corpuscle. As was explained on page 309, the older asexual and the segmenting stages of the estivo-autumnal parasite rarely appear in the peripheral blood.

Typical **"segmenters"** (Plate VII, Fig. 5, *c*) may present a ring of rounded segments or spores, each with a small, dot-like chromatin mass, but these regular forms are not often seen. With the tertian parasite, especially, the segments much more frequently form an irregular cluster. The pigment is collected near the center or at one side or is scattered among the segments.

Fully grown tertian and quartan **gametocytes** (Plate VII,

PLATE VII

Malarial Parasites.

Wright's stain. × 1000 (1 mm. = 1 μ).

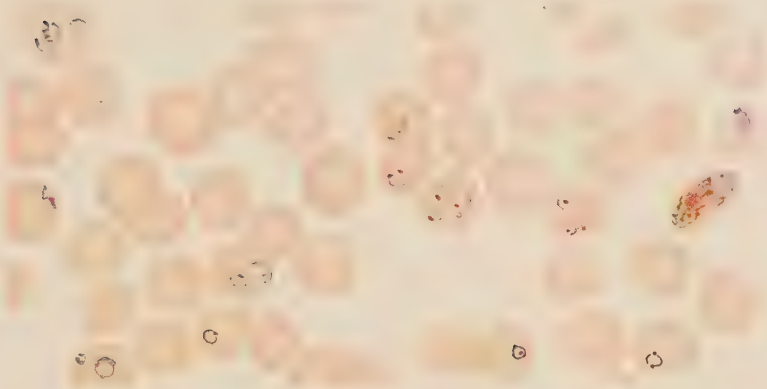


Fig. 1.—Estivo-autumnal malaria, exact reproduction of a portion of a field, showing an exceptionally large number of parasites.

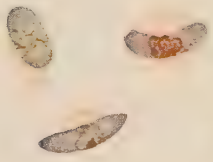


Fig. 2.—Estivo-autumnal gametocytes.

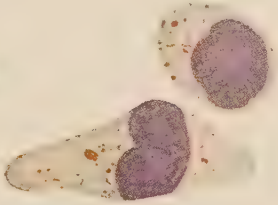


Fig. 3.—Leukocytes with engulfed pigment.

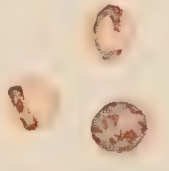


Fig 4.—Quartan parasites.

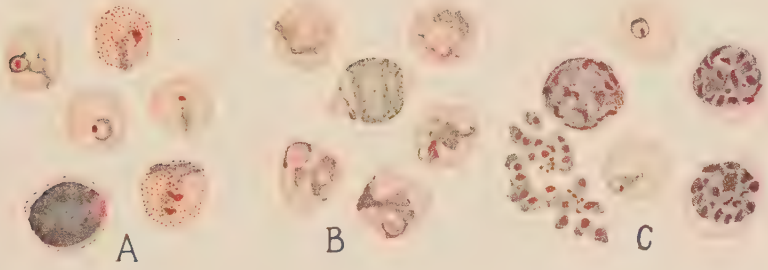


Fig. 5.—Tertian parasites: A, Eight hours after chill, showing malarial stippling, five young parasites, and one gametocyte, from two slides; B, twenty-four hours after chill, five half-grown parasites, one gametocyte; C, during chill, one presegmenter, two segmenters, a cluster of freshly liberated merozoites, and two very young parasites, from one slide.

(J. W. Rennell, pinx.)

Fig. 5, *a*, *b*) resemble the fully-grown asexual forms in general appearance, but are more compact and less irregular in shape and contain more and larger pigment granules. The female (*macrogametocyte*) is generally the larger and has more compact chromatin, usually situated near the edge of the parasite, deeper blue cytoplasm, and more pigment. The male (*microgametocyte*) stains light-blue or greenish. Its chromatin is pale and occupies a relatively large area, usually near the center. The crescentic and oval gametocytes of estivo-autumnal malaria (Plate VII, Figs. 1 and 2) are easily identified. Their length is somewhat greater than the diameter of a red corpuscle. The macrogametocyte is usually thin and more or less pointed at the poles, and the chromatin is centrally placed and surrounded by pigment granules. The microgametocyte is generally paler and thicker, with blunt ends, while its chromatin and pigment are scattered throughout the middle third of the body. The remains of the red cell often form a narrow rim around them or fill the concavity of the crescent.

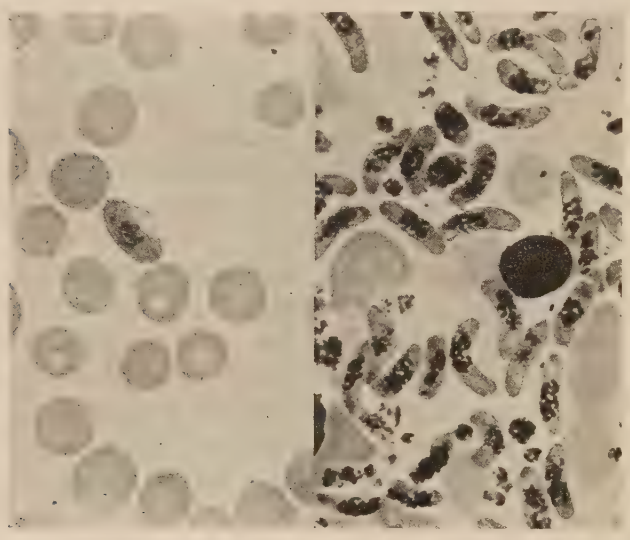
(3) **Concentration Methods for Malarial Parasites.**—When parasites are scarce they may sometimes be found, although their structure is not well shown, by the Ross-Ruge thick-smear method. This consists in spreading a very thick layer of blood, drying, placing for a few minutes in a fluid containing 5 per cent. formalin and 1 per cent. acetic acid, which removes the hemoglobin and fixes the smear, rinsing, drying, and finally staining. Carbol-thionin is very useful for this purpose. If Wright's stain be used, it is recommended that the preparation be subsequently stained for a half-minute with borax-methylene-blue (borax, 5; methylene-blue, 2; water, 100). Estivo-autumnal crescents may also be concentrated by the method given for filarial larvæ (p. 320). These older methods are, however, far inferior to the newer method of Bass and Johns, which takes advantage of the fact that parasitized red cells are lighter than the others and rise to the top of the sediment when the blood is centrifugalized at high speed. A centrifuge capable of 2500 revolutions a minute is required.

1. Draw 10 c.c. of blood from a vein (p. 222) directly into a tube containing 0.2 c.c. of citrate-dextrose solution. Mix well. The solution is made by dissolving 50 gm. sodium citrate and 50 gm. dextrose in 100 c.c. distilled water by the aid of heat.

2. Divide the blood between two centrifuge tubes and centrifugate

at 2500 revolutions per minute for the proper length of time, which is determined by the radius of the centrifuge arm and the height of the column of blood in the tube. For a centrifuge whose radius is 18 cm. the proper time is one minute for each centimeter of the blood column. Too long centrifugation will cause the corpuscles to pack so tightly that the subsequent skimming is difficult; too little centrifugation will fail to bring the parasites to the top.

3. The leukocytes and all malarial parasites (except very young forms) will now be concentrated in a layer 1 mm. thick at the top of the sediment. With a capillary pipet (Fig. 302) skim off this layer and



A B

FIG. 154.—Estivo-autumnal malaria: effect of concentration by the method of Bass and Johns: A, direct smear, averaging one crescent in eight fields; B, blood of same patient concentrated. From slides prepared by F. M. Johns. Wright's stain (photographs, × 1000).

place it, together with a like amount of plasma, in a tube about 12 cm. in length and 0.5 cm. in inside diameter. If the column of fluid exceeds 5 cm., two tubes should be used. These tubes are readily made from ordinary glass tubing.

4. Mix thoroughly and centrifugate as before.

5. With a large capillary pipet skim off the top layer of the sediment in these tubes, taking up a column of cells and plasma not exceeding 5 cm. in height.

6. Mix by forcing in and out upon a slide, and then draw the mixture into the pipet away from the tip and seal the tip in a flame. Nick with a file and break off the capillary stem above the blood column.

7. Place this slender tube in the centrifuge and revolve as before. The leukocytes will form a grayish layer upon the surface of the sediment. This and the upper portion of the erythrocyte layer contains the parasites.

8. Nick with a file and break off the capillary tube at a point 1 to 2 mm. below the bottom of the leukocyte layer.

9. With a capillary pipet whose stem will pass inside the capillary tube remove the small amount of red cells and leukocytes together with a little plasma.

10. Mix well, make smears on slides, and stain with Wright's stain in the usual way.

The authors of this method claim that 90 per cent. of the parasites in 10 c.c. of blood can be collected upon one slide. While it is not to be expected that such remarkable concentration can be attained without considerable experience, yet the method will yield good results at the first trial if the directions are carefully followed. Best results are obtained with estivo-autumnal crescents and adult tertian and quartan parasites. The very young parasites do not concentrate well, if at all. A decided advantage over the other methods is the fact that parasites and all blood-cells are perfectly preserved and stain exactly as they do in ordinary smears (Fig. 154 and Plate VI).

Fig. 155.—Filarial larva in blood (photograph, × 300). The larva is contracted, hence appears somewhat less slender than is usual. (From Beattie and Dickson's A Text-book of General Pathology, by kind permission of William Heinemann, Publisher.)

4. Filarial Larvæ (Fig. 155).—A description of the filariæ whose larvæ appear in the blood will be found on page 502. Owing to the remarkable periodicity of their appearance in the peripheral circulation, the most favorable time of day for the examination for microfilariæ will depend upon the species.

When numerous, they are easily found in fresh unstained blood. A rather large drop is taken upon a slide, covered, and examined with a low power. They can be located by the commotion which

their active motion produces among the corpuscles. This motion consists almost wholly in apparently purposeless lashing and coiling movements, and continues for many hours or even days if the preparation be ringed with vaselin and kept in a cool place. After a short time their movements clear open spaces among the corpuscles and these aid in locating the parasites. If desired, stained smears of the blood may be prepared in the usual way or by the Ross-Ruge method (p. 317). When the microfilariæ are scarce the following method is efficient:

Receive about 1 c.c. of blood from a puncture of the ear or finger into 5 c.c. of 2 per cent. acetic acid. Mix well and centrifugalize. Spread the sediment, which is not abundant, upon slides and examine in the moist state or after drying, fixing, and staining. Hematoxylin is a good stain for the purpose.

The number of microfilariæ in capillary blood is said to be distinctly higher than in that obtained from a vein.

5. Larvæ of Trichinella Spiralis.—The worm and its life history are described on page 512. In 1909 Herrick and Janeway demonstrated that diagnosis of trichiniasis can frequently be made by detection of the larvæ in the blood during their migration to the muscles. Of the examinations which have been reported since that time about one-half have been positive. The earliest time at which the embryos were found was the sixth day after the onset of symptoms; the latest, the twenty-second day.

The approved method is the same as that given above for microfilariæ, except that 5 or 10 c.c. of blood from a vein (p. 222), and a correspondingly larger quantity of acetic acid solution are required. The larvæ are not difficult to recognize. They are about 125 μ long and 6 μ broad.

XI. TESTS FOR RECOGNITION OF BLOOD

The recognition of red blood-corpuscles microscopically is the surest and simplest means of detecting the presence of blood. In most pathologic material, however, the corpuscles are too much disintegrated for recognition with the microscope, and one has to rely upon a test for hemoglobin or its derivatives. Of such tests, those given in this section are probably the best. Each is reliable within its own sphere, but each has its limitations. The guaiac, benzidin, and similar tests are reliable only when *negative*. When, however,

proper care is taken to exclude fallacies, they are the most useful and reliable tests for clinical purposes, although they could not be accepted medicolegally. The hemin test is reliable only when *positive*. The spectroscope offers perhaps the most simple and dependable means of identifying blood-pigment, but, except under favorable conditions, it is not adapted to the detection of traces. Its particular field lies in distinguishing between the various hemoglobin derivatives.

The only reliable test for human blood as distinguished from that of animals is the precipitin test described on page 578.

1. Guaiac Test.—The technic of this test has been given (p. 161). It may be applied directly to a suspected fluid, but in order to avoid other substances which might cause the reaction the following procedure is advised: Remove fat if present (for example, in feces) by shaking with an equal volume of ether and discarding the ether. It is necessary to make sure that the original fluid is not strongly acid in reaction, otherwise the blood pigment may go into solution in the ether used for the fat extraction and be unwittingly discarded. Add 3 or 4 c.c. of glacial acetic acid to about 10 c.c. of the fat-free fluid; shake thoroughly with an equal volume of ether; decant, and apply the test to the ether. Should the ether not separate well, add an equal volume of alcohol and mix gently. It should then separate nicely. When the amount of blood is very small the ether may be concentrated by evaporation, or it may be completely evaporated and the residue taken up in a few drops of water, which is then tested. In case of dried stains upon cloth, wood, or other material dissolve the stain in distilled water and test the water, or press a piece of moist blotting-paper against the stain and touch the paper with drops of the guaiac and the turpentine successively. The test may be applied to microscopic particles by running the reagents under the cover-glass.

The **benzidin test** (p. 161) is similar to the guaiac test and has the same fallacies, but is distinctly more sensitive.

2. Teichmann's Test.—This depends upon the production of characteristic crystals of *hemin*. It is not sufficiently delicate to detect the minute quantities of blood with which we frequently have to deal in the clinical laboratory, but, when positive, it is absolute proof of the presence of blood. A number of substances—lime, fine sand, iron rust—interfere with production of the crystals;

hence negative results are not always conclusive. Dissolve the suspected stain in a few drops of physiologic salt solution upon a slide. If a liquid is to be tested, evaporate some of it upon a slide and dissolve the residue in a few drops of the salt solution. Let dry, apply a cover-glass, and run glacial acetic acid underneath it. Heat *very gently* until bubbles begin to form, replacing the acid as it evaporates. Allow to cool slowly. When cool, replace the acid with water, and examine for hemin crystals with 16-mm. and 4-mm. objectives. The crystals are dark brown rhombic plates, lying singly or in crosses, and easily recognized (Fig. 156). Failure

FIG. 156.—Hemin crystals obtained in two tests for blood by Teichmann's method. Note the great difference in size of crystals formed under slightly different conditions (photographs, × 250).

to obtain them may be due to too much salt, too great heat, or too rapid cooling. If not obtained at first, let the slide stand in a warm place, as upon a hot-water radiator, for an hour, replacing the acid as it evaporates.

3. Spectroscopic Method.—Spectrum analysis depends upon the fact that solutions of many substances, when held so as to intercept the light entering the spectroscope, will absorb certain colors, thus causing dark bands to appear at definite locations in the spectrum. A small direct-vision instrument meets all ordinary requirements and may be recommended as a useful addition to the

regular laboratory equipment. The form with a side mirror and reflecting prism (Fig. 157) which gives two spectra side by side is most convenient. Before use, the width of the slit should be so adjusted and the eye-piece so focused that Fraunhofer's lines (Fig. 158, B, C, D, E, b, F) are clearly seen, since it is by means of these lines that the absorption bands are located. The examination is best made by daylight. With artificial light the Fraunhofer lines do not appear. The solution under examination may be held in a test-tube or small beaker. If a test-tube be used, only 1 to 3 c.c. will be required. *The solution must be made absolutely clear* by filtration, or, in case the quantity is very small, by centrifugation.

The **treatment of the suspected material** will depend upon its condition and the purpose of the examination:

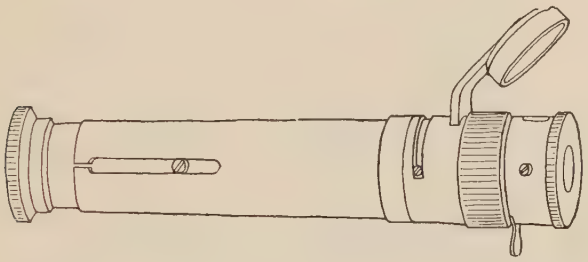


FIG. 157.—Small direct-vision spectroscope with side mirror. (About natural size.)

1. When *fresh blood* is studied for oxyhemoglobin or methemoglobin a large drop from a skin puncture is received in 1 or 2 c.c. of water in a test-tube and cautiously diluted to the point where the bands become distinct. The optimum dilution is much less for methemoglobin than for oxyhemoglobin.

2. *Urine* and *other fluids* suspected to contain blood may be cleared by filtration and examined directly. When this proves unsatisfactory, as is often the case owing to persistent cloudiness, to the presence of other pigments which darken the whole spectrum, or to the small amount of blood present, the blood pigment in 200 to 500 c.c. of the unfiltered fluid should be extracted as follows: Add a little white of egg if the fluid is not already sufficiently albuminous, boil, acidify, centrifugalize, remove supernatant fluid, and treat the sediment as described for feces in the following paragraph.

3. *Feces*, *gastric contents*, and other material should be treated with glacial acetic acid and extracted with ether as described under the

guaiac test (p. 321). Blood-pigment is thus changed to acid hematin, which is taken up by the acidified ether, giving a clear solution suitable for spectroscopic examination. If the ether does not take up the blood-pigment well, a little more acetic acid should be added. In order that the solution may not be too dilute to show the bands, a less amount of

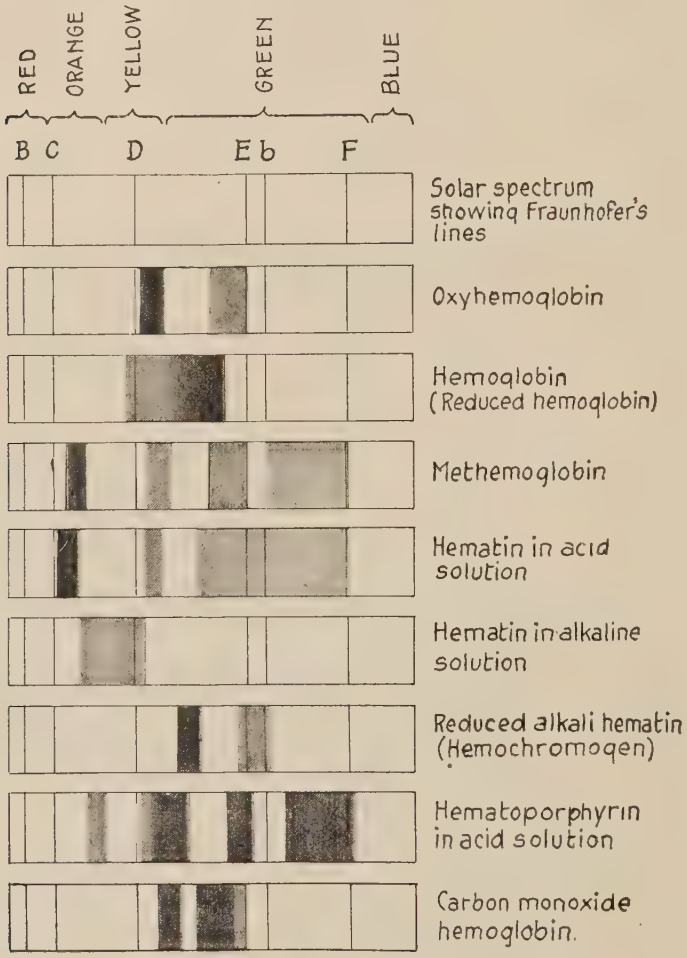


Fig. 158.—Absorption spectra of hemoglobin and its derivatives.

ether than is recommended for the guaiac test may be used, or the ether extract may be concentrated by evaporation.

When the result is in doubt the acid hematin may be transformed into the more easily identified hemochromogen as follows: Render the ethereal extract alkaline with strong ammonia, cooling if necessary, mix

well, and let stand until the fluids separate. The ammonia will contain alkali hematin. By means of a pipet transfer it to another test-tube and add a few drops of fresh yellow ammonium sulphid or Stokes' reagent.[1] Any precipitate may be removed by centrifugalization. The bands of hemochromogen should appear at once.

4. *Stains of blood dried on clothing*, and so forth, should be dissolved in 1 or 2 c.c. of 10 per cent. caustic soda solution, heated to a point just short of boiling, cooled, and treated with a few drops of ammonium sulphid or Stokes' reagent. The solution is then examined for the characteristic bands of hemochromogen.

5. In *very old blood-stains* the hemoglobin may have been transformed to the iron-free pigment hematoporphyrin, which is very resistant to solution. It will usually dissolve in strong sulphuric acid. It has been advised to place a few small bits of the dry stain on a slide in a drop of concentrated sulphuric acid, to apply a cover, and rub the bits of blood between slide and cover. Enough may go into solution to admit of spectroscopic examination. Particles of wood, cloth, or other organic material which might blacken the acid should be avoided.

The **characteristic absorption spectra** of the more important hemoglobin derivations are as follows:

1. **Oxyhemoglobin** is present only in comparatively fresh blood. It gives two dark bands between the lines D and E, the one nearer D being the stronger. In concentrated solution these unite to form a single broad band. Upon addition of a few drops of fresh ammonium sulphid, or, much better, Stokes' reagent, the spectrum changes to that of reduced hemoglobin.

2. **Hemoglobin** (also called *reduced hemoglobin*) gives a single broad band between D and E. By shaking with air it is changed to oxyhemoglobin, whose bands in the same dilution are more distinct.

3. **Methemoglobin** occurs in the circulating blood under the conditions which have been described (p. 233). It may also be found in urine and in hemorrhagic cyst fluids. In neutral or faintly acid solution its most characteristic band is situated between the lines C and D. Two less distinct bands lie between D and E, possibly due to accompanying oxyhemoglobin, and a broad one beyond E; but these are usually not clearly seen. The blood must be diluted cautiously, as it is easy

[1] Stokes' reagent consists of ferrous sulphate, 2 gm.; tartaric acid, 3 gm.; water, 100 c.c. When needed for use take a few cubic centimeters in a test-tube and add strong ammonia drop by drop until the precipitate which forms at first has entirely dissolved.

to pass the point where the characteristic band is most distinct. Upon addition of a few drops of fresh ammonium sulphid or Stokes' reagent, methemoglobin is changed to reduced hemoglobin with its single broad band. This will serve to distinguish it from acid hematin.

Methemoglobin can be prepared for purposes of comparison by diluting 2 drops of blood with 20 drops of water, adding 1 or 2 drops of strong potassium ferricyanid solution, and shaking. The solution turns chocolate brown, and may then be diluted until the characteristic band is distinct.

4. **Hematin** may be formed through the action of acids or alkalies, as in gastric and intestinal bleeding. It is sometimes found in old extravasates, in the urine, and elsewhere. It is insoluble in water or weak acids, readily soluble in acidified ether and weak alkalies.

As seen in Figure 158, the absorption bands of hematin in *acid solution* ("acid hematin") are somewhat similar to those of methemoglobin. That between C and D is most definite and charactertistic; the others may not be clearly seen. In contrast to methemoglobin, the addition of ammonium sulphid or Stokes' reagent does not produce the spectrum of reduced hemoglobin, but rather (if the solution has been sufficiently alkalinized to produce alkali hematin) that of hemochromogen.

Hematin in *alkaline solution* ("alkali hematin") gives a rather indefinite broad band between C and D. Its presence may be confirmed by adding a few drops of ammonium sulphid or Stokes' reagent. The solution becomes brighter red in color, and the spectrum changes to the more easily identified one of hemochromogen.

5. **Hemochromogen,** also called *reduced alkali hematin*, gives a narrow, very distinct band between D and E, and if not in too dilute solution, a fainter band between E and b. This is one of the most definite and characteristic of the blood-pigment spectra.

6. **Hematoporphyrin** is an iron-free hemoglobin derivative which may occasionally be present in the urine, especially in sulphonal poisoning (p. 162) and in very old dried blood. It does not respond to the guaiac or hemin test. It is soluble in strong sulphuric acid. Its absorption spectrum is shown in Figure 158.

For purposes of comparison it can be prepared by adding a drop of blood to 2 or 3 c.c. of concentrated sulphuric acid.

7. **Carbon monoxid hemoglobin,** which appears in the circulating blood in carbon monoxid poisoning, gives two bands very like those of oxyhemoglobin, but somewhat nearer the violet end of the spectrum. In contrast to oxyhemoglobin, addition of ammonium sulphid or Stokes' reagent leaves these bands unchanged. Owing to the small quantity usually present in poisoning the chemical test is preferable for its detec-

tion (p. 233). Carbon monoxid hemoglobin can be prepared for purpose of comparison by causing illuminating gas to bubble through an aqueous solution of blood in a test-tube.

XII. THE ISOHEMAGGLUTINATION GROUPS

Untoward results which sometimes follow transfusion of blood are now known to be due in most instances to hemolysis or agglutination of either the donor's or recipient's blood-corpuscles, or both. By a simple test, it is possible to ascertain whether the blood of any individual is suitable in this respect for transfusion into the veins of a given patient. This is known as "matching bloods," and it should always be done when transfusion is contemplated. The two factors to be considered are hemolysis and agglutination; but since hemolysis does not occur without agglutination, it is sufficient in practice to test for agglutination only. Blood matching is also important in selecting donors for skin-grafts.

There are two factors involved in isohemagglutination, an agglutinin in the plasma and an agglutinogen in the corpuscles which renders them agglutinable. It is an interesting fact that in respect to the presence or absence of these, every adult falls into one of several definite groups. For many years four groups, designated for convenience, I, II, III, and IV, have been recognized. To explain this grouping it is necessary to assume the existence of two separate agglutinins *a* and *b*, and the corresponding agglutinogens, *A* and *B*. A blood with a given agglutinin will agglutinate corpuscles which contain the corresponding agglutinogen, and will not affect other corpuscles. There can be no agglutination between individuals in the same group. The four groups and their interrelationships are well shown by the simple chart devised by Sanford (Fig. 159). The figures there set down for the percentage of individuals in the different groups are those of Moss and are only approximate. They vary somewhat with different races.[1]

While all individuals can be placed in one of the four groups, it has been shown by a number of observers that there are subgroups also, evidenced by the fact that occasionally blood is found with an agglutinin in its serum that acts on the corpuscles of the

[1] Hirschfeld, L., and Hirschfeld, H.: Serological Differences Between the Blood of Different Races, Lancet, vol. 2, p. 675, October, 1919. Ottenberg, Reuben: A Classification of Human Races Based on Geographic Distribution of the Blood Groups, Jour. Am. Med. Assn., vol. 84, p. 1393, May, 1925.

blood of certain individuals that are really in the same group. While these agglutinations are more evident at lower temperatures than 37° C., thus differing from the reactions of the four major groups, there is strong suggestion that severe after-effects in some transfusions might be accounted for on this basis. A complete list of references on this subject can be found in a recent article by Landsteiner and Witt.[1]

The system of numbering the four standard groups which is followed in this book is that of Moss which has been adopted by the American

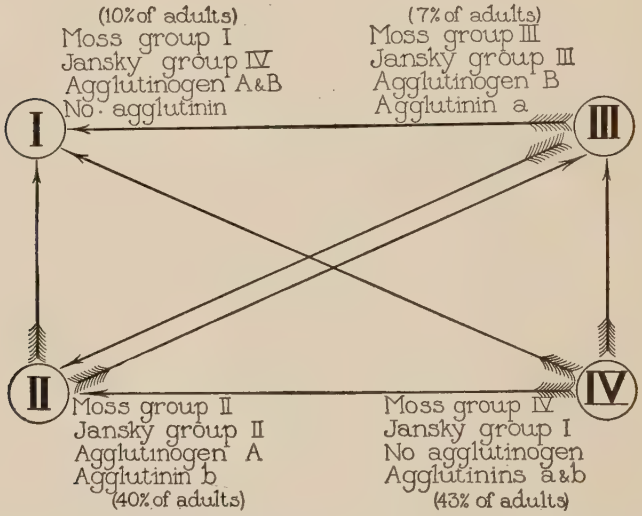


FIG. 159.—Diagram showing the interrelation of the four iso-agglutination groups of Moss (after Sanford). The serum of any group will agglutinate the corpuscles of those groups toward which its arrows point. Thus, serum of an individual belonging to Group IV will agglutinate red corpuscles belonging to any other group, while the serum of Group I lacks agglutinating power. Several subgroups have been discovered.

Society of Clinical Pathologists. Jansky's system, which *interchanges Groups I and IV*, has been approved on grounds of priority by a joint committee of the American Association of Immunologists, the Society of American Bacteriologists, and the Association of Pathologists and Bacteriologists. The Moss system has, however, become so firmly established in clinical work that a change would lead to much confusion and probably danger to life in many transfusion cases. The use of

[1] Landsteiner, Karl, and Witt, D. H.: Observations on Human Iso-agglutinins, Proc. Soc. Exp. Biol. and Med., vol. 21, p. 389, 1923–1924.

two systems side by side would be deplorable. As a result of the discovery of new groups mentioned above it is possible that the arbitrary numbers will ultimately be discarded and that the groups will be designated according to their agglutinins and agglutinogens. Thus, Group I (Moss) may be designated Group AB; Group II, Ab, and so forth. There has very recently been a well-supported proposal to name the groups by their agglutinogens: AB, A, B, and O for the Moss groups, I, II, III, and IV, respectively.

The group to which an individual belongs is an inherited characteristic which appears to follow Mendel's law, although it does not usually become fully established until after the period of infancy and sometimes not until the third year. The corpuscles show the group characteristic earlier than the serum. There are, however, iso-agglutinins and agglutinogens in the blood of many infants at birth and probably before; and this, through leakage of fetal blood into the mother's circulation, has been suggested by McQuarie as a possible etiologic factor in eclampsia, when the mother and child belong to different groups.

It is probable that the grouping may in some cases be utilized medicolegally for determining illegitimacy of children The most recent theory, advanced independently by Bernstein and by Furuhata, is that blood-groups are inherited as a series of three allelomorphs. Two dominants A and B, and a recessive R, are considered as the basis of the four groups. According to this theory paternity cannot be proved, but the impossibility of being the parent of a child in question might be established in some instances.[1]

While it is generally held that an individual's grouping never changes, a few apparent exceptions to the rule, mostly among persons who have successfully withstood acute infections or among anemic patients who have recently been transfused, have been reported. In most such cases, at least, it is probable that an agglutinin which had formerly been too weak for detection by the usual test became much stronger, thus causing an apparent change of group if only the agglutinating power of the serum was studied. This fact emphasizes the importance of making iso-agglutination tests each time transfusion is undertaken, even when the blood for a given patient is taken repeatedly from the same donor.

In an extremely few reported cases an individual's serum was capable of agglutinating his own corpuscles when cooled in the ice-box or even at room temperature. This power is absent at body temperature. The "auto-agglutinin" is quite distinct from the iso-agglutinins.

[1] "Current Comment," Jour. Amer. Med. Assoc., vol. 87, p. 1834, November, 1926. Bernstein, F.: Ztschr. f. Indukt. Abstammungs und Vererbungsl., vol. 37, p. 237, 1925. Furuhata, T.: Tenth Congress of Japanese Forensic Medicine, 1925.

When transfusion is undertaken, the blood should be secured from an individual belonging to the same group as the patient. If such a donor cannot be found, as may easily happen if the patient belongs to either of the small groups, I or III, blood belonging to another group may safely be used *provided that serum of the patient does not agglutinate the corpuscles of the donor, and that the blood be introduced very slowly*. Thus blood of Group IV (Moss) may in emergencies be used for patients of any other of the four groups. The reason for this is found in the fact that although the donor's blood has the power of agglutinating the recipient's corpuscles in a test-tube or on a slide, yet in actual transfusion, slowly carried out, the blood which is introduced mixes at once with the recipient's blood and is so greatly diluted that its power to injure the recipient's corpuscles is greatly reduced, if not completely lost. If, upon the other hand, blood of Groups I, II, or III be used for a Group IV patient, the introduced corpuscles are subjected to practically the full strength of the patient's blood, and all are agglutinated or hemolyzed with disastrous results for the patient. When transfusion of patients with abnormally fragile red corpuscles—as in hemolytic jaundice—is undertaken, use of a donor of the same group is obligatory; and, moreover, the donor's blood should be matched directly with the patient's before each transfusion.

Technic of Blood Matching.—1. Obtain the following from each of the two persons whose blood is to be matched:

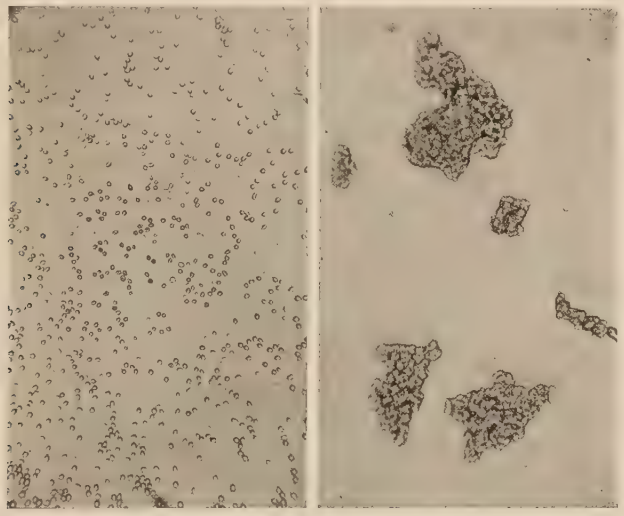
(*a*) *Red-cell Suspension.*—Puncture finger or ear, and let a large drop of blood fall directly into a small test-tube containing 1 c.c. of a 1 per cent. solution of sodium citrate in 0.85 per cent. salt solution. Mix gently by inverting a few times. It will often be found more convenient to use the "white" pipet of the hemacytometer, making a 1 : 20 dilution which is the optimum for the red-cell suspension.

(*b*) *Serum.*—Obtain a few drops of blood in a small tube or Lyon capsule (Fig. 285). As soon as coagulation has taken place, gently loosen the clot from the wall of the tube. Let stand until serum has separated well. Separation of serum can be hastened by centrifugation.

2. Make vaselin rings on two slides. In one mix 1 large drop each of the patient's serum and the suspension of the donor's corpuscles; in the other mix 1 large drop each of the patient's corpuscles and the donor's serum. Label the slides with a wax pencil. The fluids are best transferred to the slide by means of a capillary pipet (Fig. 302); a platinum loop is too small.

3. Keep the slides at room-temperature and every few minutes remix corpuscles and serum by tilting the slide.

4. At intervals examine for agglutination of red corpuscles with a low-power objective. When agglutination takes place the corpuscles gather into dense irregular clumps (Fig. 160). These are nearly always so large as to be seen with the unaided eye as brick-red granules, best viewed over a sheet of white paper; and many workers prefer not to use the microscope at all. Clumping is usually well marked within a few minutes, but it is safe to allow twenty minutes. If it does not occur within this time, it will not occur at all.



A B

FIG. 160.—Matching bloods for transfusion: A, Corpuscles of a patient with serum of a prospective donor; no agglutination. B, Serum of patient with corpuscles of prospective donor; strong agglutination. The blood of the donor is therefore unsuited for use in this case (photographs, × 100).

Sources of Error.—An important source of error is rouleau formation, which may or may not occur. Although the clumps are usually very small, this is not always easy to differentiate without close observation with the 4 mm. objective. In the case of rouleau formation the corpuscles can be seen to lie in rows within the groups (Fig. 93). Frequent remixing of the corpuscles and serum, as above directed, tends to break up rouleau and to favor agglutination.

A still more important source of error lies in the fact that sera differ in their agglutinating titer and the same individual's serum may vary from time to time. This is particularly true in the case of Group III serum. It has recently been shown that diet has an influence. Very

rarely the agglutinating power may be so low as to be overlooked in a routine test. When results are in doubt the test should be repeated with two or three times the usual amount of serum. Especial care must be exercised in matching the blood of children because the group characteristics may not yet be fully developed.

To Determine the Group to which an Individual Belongs.—This is called **blood typing.** As has been indicated above, it is sufficient in a given case to test the blood of a series of prospective donors until one is found which matches the patient's blood, and such direct matching is obligatory in certain cases. In hospital work, upon the other hand, it will be found much more convenient to determine in advance the grouping of a number of individuals who may be willing to serve as donors upon occasion. When an emergency arises, it is then only necessary to find the group to which the patient belongs in order to know at once the appropriate donor, a procedure which does not require more than fifteen minutes; but even here there should be a direct test of the donor's corpuscles against the patient's serum before the transfusion is done.

The group to which an individual belongs is easily ascertained by testing his serum and corpuscles against the corpuscles and serum of an individual known to belong to Group II or III, using the simple method described above. Interpretation of results is made clear by Figure 159. If, for example, the unknown blood agglutinates Group II blood and is not agglutinated by it, then the unknown must belong to Group IV (Moss).

The same end may be accomplished by testing the corpuscles of the unknown against sera of both Groups II and III. As practised by Vincent, a drop of each of the sera is placed on a slide, one at each end, and a drop of the suspension of unknown corpuscles or a loopful of the whole blood obtained directly from a skin puncture is mixed with each. Here also reference to Figure 159 will make the interpretation clear. Agglutinating sera, if kept sterile, will remain active for months and may be kept on hand in small glass capsules (Fig. 284) the ends of which are to be sealed in the flame. It will also remain active for many months if dried on glass or paper, or if preserved with 0.5 per cent. of phenol or 0.25 per cent. of trikresol.

In view of the existence of the subgroups already mentioned, it is desirable in typing to test both corpuscles and serum of the unknown against serum and corpuscles of a number of individuals belonging to different groups. In this way anomalous grouping is more likely to be discovered.

XIII. CHEMICAL EXAMINATION OF THE BLOOD

Until recently the chemical study of the blood, aside from the approximate estimation of hemoglobin which is detailed in an earlier

section of this chapter, has interested the biochemist rather than the clinician. The past few years, however, have brought an actively growing interest in the chemical composition of the blood in disease. Improvements in technical methods have been introduced almost weekly, and so many facts of clinical application have been gathered that certain chemical examinations now play an important rôle in clinical medicine. Among the more useful of these are estimations of non-protein nitrogen, urea, uric acid, and creatinin in kidney diseases; of sugar in diabetes, renal glycosuria, and certain other disorders; of carbon dioxid combining power in acidosis, and of bile-pigment in jaundice. These are discussed in the following pages. Others, which have less practical value and for which the reader is referred to special works upon blood chemistry, are cholesterol, phosphates, and enzymes.

Obtaining Blood for Chemical Examination

Blood for chemical examination is obtained from a vein (p. 222), most conveniently in a 1-ounce "powder mouth" bottle with stopper and tubes as shown in Figure 99. The receiving bottle must contain a small amount, about 20 mg., of potassium oxalate to prevent coagulation. The oxalate is best introduced by placing in the bottle 2 drops of a 20 per cent. solution of chemically pure neutral potassium oxalate and drying in a hot-air sterilizer. For uric acid determinations Folin advises use of 10 mg. of lithium oxalate. Immediately after the blood is added the bottle is stoppered and well shaken to insure proper mixing with the oxalate. Shaking, however, is to be avoided when the CO_2-combining power is to be estimated. The amount of blood required will depend upon the estimations to be carried out. From 6 to 10 c.c. will usually suffice.

The concentration of various constituents of the blood is materially altered for a time following meals; and it is therefore necessary, for the sake of uniform results, to obtain the blood after a twelve-hour fast—that is, before food is taken in the morning. Unless a preservative is used the examination should be started on the same day, preferably within an hour, and the blood should be kept on ice in the interval.

Sander[1] has recommended as a preservative a mixture of 0.01 gm.

[1] Sander, F. V.: The Preservation of Blood for Chemical Analysis, Jour. Biol. Chem., vol. 58, p. 1, November, 1923.

of sodium fluorid and 0.001 gm. of thymol for each cubic centimeter of blood. The non-protein nitrogen content remains constant with this mixture for six days, and the urea, uric acid, creatinin, creatin, and sugar are preserved for as long as fourteen days. The mixture is prepared by powdering 1 part of thymol with 10 parts of sodium fluorid in a mortar, and passing several times through a 100-mesh sieve. A weighed amount, sufficient for the quantity of blood that is to be taken (.011 gm. for each c.c.), is then put in each bottle. The sodium fluorid should be free from ammonia; 1 gm. should give no color when treated with 5 c.c. of Nessler's solution. This preservative also serves as an anticoagulant, obviating the use of oxalate

Removal of Blood Proteins.—1. By means of a pipet place a measured amount, usually 5 to 10 c.c., of the oxalated blood (p. 333) in a 200-c.c. flask, add seven times its volume of distilled water, and mix well.

2. Add an amount of 10 per cent. solution of sodium tungstate[1] equal to the volume of oxalated blood used, and mix.

3. Add very slowly drop by drop, with constant shaking, an amount of two-thirds normal sulphuric acid equal to the volume of oxalated blood used.

4. Insert a rubber stopper and give a few vigorous shakes. A dark brown coagulum should form. Should it fail to do so, coagulation is incomplete owing, probably, to use of too much oxalate. In such cases add a few drops of twice normal sulphuric acid, shake vigorously, and allow the mixture to stand for five minutes for the coagulum to change from bright red to dark brown, before filtering.

5. Filter through paper and collect the clear filtrate. Should the first that comes through be cloudy, return it to the funnel. The filtrate should show no acid when tested with Congo red paper. When uric acid is to be determined, Benedict recommends that the mixture be allowed to stand ten to twenty minutes after the sulphuric acid is added and before filtering.

Each cubic centimeter of the clear filtrate represents 0.1 c.c. of blood. It will serve for determinations of non-protein nitrogen, urea,

[1] Folin has pointed out that the sodium tungstate upon the market is not uniform. Some lots are alkaline and some acid. A satisfactory sodium tungstate gives a solution which is neutral or faintly alkaline to phenolphthalein. The sample should be discarded if it is so alkaline that more than 0.4 c.c. of decinormal acid are required to neutralize 10 c.c. of the 10 per cent. solution with phenolphthalein as indicator. Acid-reacting tungstate may be used, provided the 10 per cent. solution be brought to neutrality or very faint alkalinity with sodium hydroxid.

uric acid, creatinin, blood sugar, and other substances, and may be kept without deterioration for two days or longer if covered with a few drops of toluene, or xylene, and kept on ice.

A. Non-protein Nitrogen. Urea. Creatinin. Uric Acid

As was stated in connection with the tests of renal function, it is customary to divide the nitrogen-containing constituents of the blood into two groups, one including the proteins (albumins and globulins) and the other including the various non-protein nitrogenous substances (unutilized food derivatives, waste metabolic products, and so forth).

Because of the light which they throw on the problems of metabolism and excretion it is the non-protein group, and particularly the waste products, urea, uric acid, and creatinin, which are of chief interest from the clinical point of view. The amounts present in the blood in health are as follows:

	Per 100 c.c. of blood.
Total non-protein nitrogen	25–30 mg.
Urea nitrogen	12–15 "
Uric acid	2– 4 "
Creatinin	1– 2 "

Lower figures are infrequent and have no definite clinical import. Higher figures are usually referable to accumulation of the respective substance in the blood because of defective elimination. The subject is discussed in the sections dealing with the functional capacity of the kidneys and with laboratory findings in nephritis. Since blood urea has apparently the same significance as regards kidney function as has the total non-protein nitrogen, and since estimation of urea is much the simpler, the method for non-protein nitrogen is much less used than is that for urea. Recent studies by Behre and Benedict throw doubt upon the existence of creatinin in the blood, but the facts which have been gathered regarding the clinical significance of the determinations still stand.

The methods which follow are, for the most part, based upon the system of blood analysis devised by Folin and Wu.[1]

[1] Folin, O., and Wu, H.: A System of Blood Analysis, Jour. Biol. Chem., vol. 38, p. 81, May, 1919.

Determination of Total Non-protein Nitrogen

Method of Folin and Wu.—*Reagents.*—(*a*) Digestion mixture. To 50 c.c. of 5 per cent. copper sulphate solution add 300 c.c. of 85 per cent. phosphoric acid and mix. Add 100 c.c. of concentrated sulphuric acid and mix. For use dilute 100 c.c. of the acid mixture with 100 c.c. of water. Cover well.

(*b*) Nessler's solution (1). Stock solution of mercuric potassium iodid. Transfer 150 gm. of potassium iodid and 110 gm. of iodin to a 500-c.c. Florence flask; add 100 c.c. of water and 140 to 150 gm. of metallic mercury. Shake the flask continuously and vigorously for from seven to fifteen minutes, or until the dissolved iodin has nearly all disappeared. The solution becomes hot. When the red iodin solution has begun to become visibly pale, cool in running water, and continue the shaking until the red color of the iodin has been replaced by the green color of the double iodid. Separate the solution from the surplus mercury by decantation and washing with liberal quantities of distilled water. Dilute the solution and washings to a volume of 2 liters.

(2) Final solution. Prepare a solution of 55 gm. sodium hydroxid for each 100 c.c. distilled water. Decant the clear supernatant liquid and dilute to a concentration of 10 per cent. as determined by titration, an error of not over 5 per cent. being allowed. Transfer to a bottle 1400 c.c. of this 10 per cent. sodium hydroxid. Add 300 c.c. of the stock mercuric potassium iodid solution and 300 c.c. distilled water, making 2 liters of Nessler's reagent.

(*c*) Standard ammonium sulphate (0.47165 gm. ammonium sulphate in 1 liter water); 10 c.c. of this standard contains 1 mg. of nitrogen.

Method.—1. Transfer 5 c.c. protein-free blood filtrate (p. 334) to a dry pyrex tube graduated at 35 and 50 c.c.

2. Add 1 c.c. of the diluted acid digestion mixture and a quartz pebble. Boil vigorously over a microburner until the characteristic dense fumes nearly fill the tube (from three to seven minutes).

3. Reduce flame sharply, so the boiling is barely perceptible, cover the mouth of the test-tube with a watch-glass, and continue heating for two minutes or until the solution is nearly colorless.

4. Remove the flame and allow mixture to cool for from seventy to ninety seconds.

5. Add from 15 to 25 c.c. water.

6. Cool to room-temperature, and add water to the 35 c.c. mark.

7. Add 15 c.c. Nessler's reagent; insert clean rubber stopper, and mix. If the solution is turbid, centrifugalize a portion before comparing with the standard.

8. Prepare the standard (usually 0.3 mg. of nitrogen required); place

3 c.c. of standard ammonium sulphate (*c*) and 2 c.c. of acid digestion mixture, in a 100 c.c. volumetric flask; add 60 c.c. water, 30 c.c. Nessler's reagent, and make up to 100 c.c. Nesslerize practically simultaneously with the unknown.

9. Compare the unknown with the standard in a colorimeter. If a Duboscq colorimeter is used, set the standard at 20 mm.

Calculation:

$$\frac{\text{Reading of Standard (20)}}{\text{Reading of Unknown}} \times 30 = \text{mg. of non-protein nitrogen in 100 c.c. blood.}$$

Determination of Blood Urea

Method or Folin and Wu with Aëration.—*Reagents Required.*—All of these may be purchased ready prepared.

(*a*) Urease solution[1]: Place about 3 gm. of permutit powder in a 200-c.c. flask. Wash by shaking with 2 per cent. acetic acid, allowing to settle, and pouring off the supernatant fluid. Wash twice with distilled water in a similar manner. Add to the moist permutit 100 c.c. of 30 per cent. alcohol (35 c.c. of 95 per cent. alcohol and 70 c.c. water). Add 5 gm. jack bean meal and shake for ten minutes. Filter and store in small bottles. The solution remains good for about a month if kept on ice. Each estimation of blood urea will require 0.5 to 1 c.c.

(*b*) Pyrophosphate solution: Dissolve 14 gm. sodium pyrophosphate, U. S. P., and 2 gm. of glacial phosphoric acid in 100 c.c. distilled water. Two drops are used in each estimation to accelerate the action of urease.

(*c*) Sodium hydroxid, 10 per cent. solution. Each estimation requires 1 or 2 c.c.

(*d*) Antifoam liquid. Pure caprylic alcohol is best and requires the use of only 4 or 5 drops. Pure amyl alcohol may be substituted, but 1 or 2 c.c. must be used.

(*e*) Standard ammonium sulphate solution. (See Non-protein Nitrogen Method, Reagent *c*.)

(*f*) Nessler's reagent (Folin). (See Non-protein Nitrogen Method Reagent *b*.)

(*g*) Decinormal hydrochloric acid solution. One c.c., diluted with water, is used in the determination to catch the ammonia carried over by aëration.

[1] Instead of Folin's urease and pyrophosphate solutions here described one may use a 5 per cent. solution of the urease powder prepared by E. R. Squibb & Sons, and the Arlington Chemical Co., or the 0.025 gm. tablets prepared by Hynson, Westcott & Dunning. Two tablets broken up in 1 c.c. of water make a 5 per cent. solution. All of these have the activating phosphate already added.

Method: 1. Pipet 5 c.c. of the protein-free filtrate[1] (p. 334), representing 0.5 c.c. of blood, into a test-tube of such size that it will readily slip into cylinder A of the aëration apparatus of Myers (Fig. 161).

2. Add 0.5 to 1 c.c. of the urease solution and 2 drops of the pyrophosphate solution. The pyrophosphate is to be omitted if the urease solution is prepared as described in the footnote on page 337.

3. Let stand at room temperature for fifteen or twenty minutes, or place in a water-bath at 50° to 55° C. for five minutes. The urea is converted into ammonium carbonate.

4. Add 1 or 2 c.c. of 10 per cent. sodium hydroxid solution, mix and add 4 or 5 drops of caprylic alcohol or 1 or 2 c.c. of pure amyl alcohol.

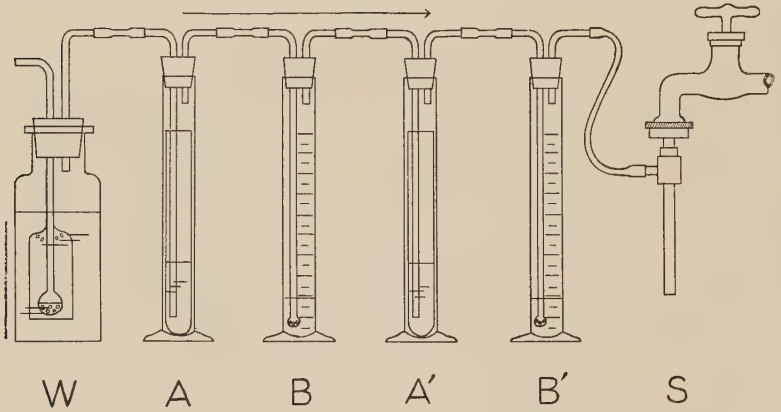


Fig. 161.—Aëration apparatus for estimation of urea and ammonia: W, Bottle containing dilute sulphuric acid to remove ammonia from the air which passes through the apparatus, A, A′, cylinders in which the test fluids are placed; B, B′, cylinders with decinormal acid to receive the ammonia; S, suction.

Immediately place the test-tube in cylinder A (Fig. 161) and adjust the stopper, taking care that the end of the delivery tube is well below the surface of the fluid.

5. In graduated cylinder B place 1 c.c. of decinormal hydrochloric acid and 15 or 20 c.c. of water. Adjust the stopper with the end of the delivery tube reaching nearly to the bottom of the cylinder.

6. Start the suction, allowing the air to pass only very gently for the first few minutes, later as actively as the apparatus will stand. Continue aëration for thirty to forty-five minutes.

7. When aëration is completed, add 2.5 c.c. of Nessler's solution, to

[1] Precipitation of proteins is not necessary and is done only when other substances are to be determined in the same sample of blood. When only urea is to be determined it is more convenient to use the whole blood as follows: Take 1 c.c. of the oxalated blood, add 9 c.c. of water, mix well, and use 5 c.c. in Step 1 above.

the acid in cylinder B, fill to the 25 c.c. mark with distilled water, and mix well.

8. Prepare the standard color solution, containing 0.3 mg. of nitrogen as follows: In a 100-c.c. volumetric flask take exactly 3 c.c. of the standard ammonium sulphate solution, about 70 c.c. of water, and 10 c.c. Nessler's reagent. Make up to the 100 c.c. mark with water, and mix well. *The standard and the unknown should be Nesslerized as nearly simultaneously as practicable.*

9. Compare the unknown with the standard in a colorimeter, calculate the amount of the urea nitrogen in the 0.5 c.c. of blood used, and from this the amount of urea nitrogen in 100 c.c. of the blood.[1] The details of the calculation vary with the colorimeters used, but depend upon the fact that 25 c.c. of the unknown represent 0.5 c.c. of blood, while 100 c.c. of the color standard contain 0.3 mg. nitrogen. With the Duboscq type of colorimeter the following formula may be used:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 15 = \text{mg. urea nitrogen in 100 c.c. blood.}$$

While the method as given above is well suited to the needs of most clinical laboratories, a variation which is given preference by Folin and Wu is widely used. This consists in carrying the ammonia from the blood filtrate into the acid by means of distillation instead of aëration. The tube containing the blood filtrate and urease is connected to a receiving tube in a manner similar to that shown in Figure 51. The method is exactly the same as that given above, except that Steps 4 to 7 inclusive are changed to read as follows:

4. To the digested blood-filtrate add 2 or 3 drops of paraffin oil and 1 c.c. of 10 per cent. sodium hydroxid, and insert the stopper.

5. Quickly place 1 c.c. of decinormal hydrochloric acid and about 1 c.c. of distilled water in the receiving test-tube and connect with the delivery tube. The end of the delivery tube must extend below the surface of the acid. The receiving test-tube should have a 25 c.c. mark.

6. Boil moderately over a microburner for four minutes. At the end of this time lower the receiving test-tube so that the tip of the delivery tube touches its wall near the top, and boil vigorously for another minute. Rinse the end of the delivery tube with distilled water into the receiving tube.

7. Cool the acid, which now contains the ammonia, dilute to about 20 c.c. with distilled water, add 2.5 c.c. Nessler's reagent, make up to exactly 25 c.c. with distilled water, and mix.

[1] Urea and urea-nitrogen figures should not be confused. To find the amount of urea multiply the urea-nitrogen figures by 2.14.

Van Slyke and Cullen Modification of Marshall Urease Method. —(Also page 123.)

Reagents.—(*a*) Urease solution, 10 per cent. urease powder in distilled water.

(*b*) Antifoam solutions.

1. Rosin, 20 gm.; turpentine 80 c.c. To be used in the tubes containing blood.

2. Amyl alcohol, 30 c.c.; kerosene, 70 c.c. To be used in the tubes containing acid.

(*c*) Potassium carbonate, 100 gm. in distilled water, 100 c.c.

(*d*) Indicator, 1 per cent. solution alizarin red S (sodium alizarin sulphonate), in distilled water.

(*e*) Volumetric solutions, fiftieth normal sulphuric, or hydrochloric acid, and fiftieth normal alkali.

Method.—Pipet 3 c.c. whole oxalated blood into a 100 c.c. pyrex test-tube.

2. Add 1 c.c. urease solution. (*a*) Place tube in water-bath at 50° to 55° C. for fifteen minutes.

3. Add 1 to 2 c.c. rosin antifoam solution (b, 1).

4. Place 10 c.c. fiftieth normal acid in acid tube.

5. Add 1 c.c. amyl alcohol antifoam solution (b, 2) to this tube.

6. Connect apparatus with suction pump and start aëration of blood tubes into acid tubes.

7. Add 10 c.c. saturated solution potassium carbonate, (*c*) to blood-tube, stopper quickly and tightly, continue aëration for forty-five minutes, driving off all ammonia.

8. Determine the excess of acid by titrating the contents of the acid-tube with fiftieth normal sodium hydroxid, using 1 drop of alizarin red indicator (d).

9. Blank tubes, without blood, are set up, aërated and titrated, to determine the amount of ammonia in the reagents.

Calculation.—Number cubic centimeters of fiftieth normal sodium hydroxid used to titrate blank acid-tube − number cubic centimeters fiftieth normal hydroxid used to titrate blood acid-tube = number cubic centimeters of fiftieth normal acid neutralized by ammonia from the blood.

Amount of acid neutralized by ammonia from the blood × 20 × 0.467 = mg. urea nitrogen for each 100 c.c. blood.

Determination of Blood Creatinin

Reagents Required.—(*a*) Alkaline picrate solution: Saturated solution purified picric acid, 25 c.c.; sodium hydroxid, 10 per cent. solution, 5 c.c.

This solution must be freshly mixed for each test. The picric acid and the sodium hydroxid solutions keep for a long time.

(*b*) Standard creatinin solution: Dissolve 0.1 gm. creatinin, or 0.161 gm. of creatinin zinc chlorid, in 80 c.c. decinormal hydrochloric acid and make up to 100 c.c. This constitutes a stock solution from which the standard creatinin solution is made as follows: In a liter volumetric flask place 6 c.c. of the stock solution, fill to the 1000 c.c. mark with decinormal hydrochloric acid, and mix well. Preserve by adding a few drops of toluene or xylene; 5 c.c. of this standard contain 0.03 mg. creatinin.

Method.—1. Pipet 10 c.c. of the protein-free blood filtrate (p. 334) into a small flask.

2. Pipet 5 c.c. (10 c.c., 15 c.c., or 20 c.c., if high creatinin is expected) of the standard creatinin solution into another flask and dilute to 20 c.c. with water.

3. As nearly simultaneously as possible add 5 c.c. of the freshly prepared alkaline picrate solution to the first of the two flasks and 10 c.c. to the second which contains the standard. Mix well.

4. At the end of eight to ten minutes compare the unknown with the standard in a colorimeter. The reading must be completed within fifteen minutes from the time the picrate was added. The calculation will vary with the colorimeter used, but depends upon the fact that the unknown represents 1 c.c. of blood, while the standard, diluted to twice the volume, contains 0.03 mg. creatinin (or 0.06 mg. if 10 c.c. of standard creatinin were used).

With the plunger type of colorimeter the following formula may be used: $\frac{S}{R} \times \frac{C}{2} \times 100 =$ mg. creatinin in 100 c.c. of blood, S representing the reading of the standard, R the reading of the unknown, C the strength of the standard in mg. of creatinin. Thus if the standard is set at 20, and the reading of the unknown is 10 while the standard contains 0.03 mg. creatinin, then the formula gives a creatinin value of 3 mg. per 100 c.c. of blood.

Determination of Blood Uric Acid

Benedict's Method.—*Reagents Required.*—(*a*) Standard uric acid solution (Benedict and Hitchcock). In about 500 c.c. of hot distilled water dissolve 9 gm. pure crystalline hydrogen disodium phosphate and 1 gm. dihydrogen sodium phosphate. Filter if not perfectly clear. In a 1000-c.c. volumetric flask place exactly 200 mg. uric acid suspended in a few cubic centimeters of distilled water. Pour the hot phosphate solution into the flask. Agitate until the uric acid is completely dissolved and add 1.4 c.c. glacial acetic acid. Cool to room temperature,

make up to 1000 c.c. with distilled water, and mix well. Add 5 c.c. of chloroform as preservative; 5 c.c. of this solution contain 1 mg. uric acid. It remains good for at least two months.

From the above stock of uric acid solution prepare two standards for use in uric acid determinations:

No. 1. Stronger standard. Measure 25 c.c. of the above stock solution into a 500-c.c. volumetric flask, add about 250 c.c. of water and 25 c.c. diluted hydrochloric acid (concentrated hydrochloric acid 1 part, water 9 parts). Dilute to 500 c.c. and mix well. This solution contains 0.05 mg. uric acid in 5 c.c.

No. 2. Weaker standard. Proceed in exactly the same way as for the stronger standard, but use 10 c.c. of the stock solution instead of 25 c.c. This standard contains 0.02 mg. uric acid in 5 c.c. and is the one most frequently required. It remains good for two weeks.

(*b*) Sodium cyanid, 5 per cent. Dissolve 25 gm. sodium cyanid in 450 c.c. distilled water, add 1 c.c. concentrated ammonia, make up to 500 c.c., and mix. This solution must be freshly prepared once in two months.

(*c*) Uric acid reagent (Benedict). Place 100 gm. of sodium tungstate (Merck or Baker, c. p.) in a liter pyrex flask and dissolve in about 600 c.c. of distilled water. Add 50 gm. of pure arsenic acid (As_2O_5), 25 c.c. of 85 per cent. phosphoric acid, and 20 c.c. of concentrated hydrochloric acid. Boil for about twenty minutes, cool, and dilute to 1000 c.c. This reagent remains good indefinitely.

Method.—1. In a test-tube of about 18 to 20 mm. diameter place 5 c.c. of the clear, protein-free blood-filtrate (p. 334) representing 0.5 c.c. of blood, and add 5 c.c. of distilled water. Mark the tube U.

2. In a test-tube of the same diameter, place 5 c.c. of the stronger uric acid standard solution described above, and add 5 c.c. of water. Mark this tube S 1. It contains 0.05 mg. uric acid.

3. In a third test-tube place 5 c.c. of the weaker standard, and add 5 c.c. of water. Mark this tube S 2. It contains 0.02 mg. uric acid.

4. To each tube add 4 c.c. of 5 per cent. sodium cyanid solution from a buret.

5. To each tube add 1 c.c. of the uric acid reagent. Mix by one inversion and immediately place in boiling water for three minutes after immersion of the last tube. The time elapsing between immersion of the first and last tubes must not exceed one minute.

6. Remove the tubes, cool for three minutes in a beaker of cold water, and compare the unknown in a colorimeter with the standard which it more nearly matches. To avoid precipitates the comparison should be made within five minutes after removal from the cold water.

The calculation is based upon the fact that the unknown represents 0.5 c.c. of blood, while No. 1 and No. 2 standards contain 0.05 and 0.02 mg. of uric acid respectively. With the Duboscq or Denison Laboratory colorimeter and the No. 2 standard the following formula may be used:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 4 = \text{mg. of uric acid in 100 c.c. of blood.}$$

Folin's Method.—*Reagents Required.*—(*a*) Standard stock solution of uric acid. Place 0.45 to 0.5 gm. lithium carbonate in a 300-c.c. beaker. Add 150 c.c. water and heat to 60° C. Stir until dissolved. Transfer exactly 1 gm. of uric acid to a funnel on a 300-c.c. flask. Rinse this into the flask with the hot lithium carbonate solution, and shake. When the uric acid has dissolved, cool under running water and transfer to a 1 liter volumetric flask. Dilute to about 500 c.c. Add 25 c.c. of 40 per cent. formalin, shake, and acidify with 3 c.c. glacial acetic acid. Shake to remove most of the CO_2, and add distilled water exactly to the liter mark, and mix by shaking. This stock standard solution will keep for several months, but should be placed in small, tightly stoppered bottles and kept in a dark place. Each cubic centimeter contains 1 mg. uric acid.

(*b*) Diluted uric acid standard. Transfer, with an Ostwald pipet, 1 c.c. of the stock standard to a 250-c.c. volumetric flask. Fill the flask half full with distilled water, and add 10 c.c. of two-thirds normal H_2SO_4, and 1 c.c. of 40 per cent. formalin. Dilute to 250 c.c. volume and mix. The diluted standard should keep for a month; 5 c.c. contains 0.02 mg. uric acid.

(*c*) Uric acid reagent. Transfer 50 c.c. of 85 per cent. phosphoric acid, and 160 c.c. of distilled water to a 500-c.c. pyrex flask. Heat nearly to boiling and add 100 gm. of sodium tungstate. The mixture begins to boil from the heat of the reaction. Boil gently and continuously over a microburner for one hour. Close the tube with a funnel covered with a 200-c.c. Florence flask filled with water. Transfer 25 gm. of lithium carbonate to a liter beaker, add 50 c.c. of 85 per cent. phosphoric acid, and 200 c.c. of water. Boil off the CO_2, and cool. Mix the two solutions, and dilute to one liter.

(*d*) Sodium cyanid solution, 15 per cent., in tenth-normal sodium hydroxid. Let stand two weeks before using.

Method.—1. Transfer 5 c.c. of protein-free blood-filtrate (p. 334) to a test-tube graduated at the 25-c.c. mark.

2. Transfer 5 c.c. of the diluted uric acid standard (*b*) to a similar tube.

3. Add to each test-tube 2 c.c. of water, 2 c.c. of the cyanid solution (from a buret), and exactly 1 c.c. of uric-acid reagent. Mix and let stand for two minutes.

4. Heat in a boiling-water bath for eighty seconds. Cool, and dilute to 25 c.c.

5. The standard should first be read against itself in the colorimeter, and then the standard set at 20 mm., and the unknown compared with it. Readings between 40 and 10 mm. (2 to 8 mg. uric acid) are dependable.

Calculation:

$$\frac{\text{Reading of the Standard (20 mm.)}}{\text{Reading of the Unknown}} \times 4 = \text{mg. uric acid for each 100 c.c. blood.}$$

B. Blood Chlorids

The normal amount of sodium chlorid in the whole blood is 0.45 to 0.50 per cent. The plasma, however, contains 0.57 to 0.62 per cent. In nephritis, eclampsia, anemia, and at times in malignant conditions and cardiac disease, the chlorid content of the blood is above normal. In cases with fever, in diabetes, in pneumonia, and in severe toxemia with obstructions of the upper gastro-intestinal tract the blood chlorids are low. In the treatment of this last condition the administration of sodium chlorid, along with dextrose and water, has been found of value.

Many methods have been devised for the estimation of blood chlorids. One of the simplest and most satisfactory is that of Whitehorn[1] which is here given.

Method of Whitehorn (slightly modified).—*Reagents Required.*—(*a*) Silver nitrate solution, $\frac{M}{35.46}$; 1 c.c. = 1 mg. of Cl. Dissolve 4.791 gm. of C. P. silver nitrate in distilled water, and dilute to 1 liter. Preserve in a brown bottle.

(*b*) Potassium sulphocyanate solution, $\frac{M}{35.46}$. Dissolve 3 gm. of KCNS in 1 liter of distilled water. Standardize nitrate solution (*a*). To do this add 5 c.c. concentrated nitric acid solution (*d*) to 5 c.c. of the silver nitrate solution. Mix and let stand five minutes. Then add 0.3 gm. ferric ammonium sulphate (*c*), and titrate with the sulphocyanate solution.

[1] Whitehorn, J. C.: Simplified Method for the Determination of Chlorids in Blood or Plasma, Jour. Biol. Chem., vol. xlv, p. 449, February, 1921.

(*c*) Powdered ferric ammonium sulphate.

(*d*) Concentrated nitric acid; specific gravity 1.42; chlorid free.

(*e*) Ten per cent. sodium tungstate solution.

(*f*) Two-thirds normal sulphuric acid.

Method.—1. Transfer 2 c.c. blood-plasma to a small Erlenmeyer flask; dilute with 16 c.c. (8 volumes) of distilled water. Add 1 c.c. (one-half volume) of 10 per cent. sodium tungstate (*e*), and 1 c.c. of two-thirds normal sulphuric acid (*f*); shake. Let stand ten minutes, and filter.

2. Transfer 10 c.c. of the filtrate into an Erlenmeyer flask. Add 5 c.c. of the standard silver nitrate solution (*a*). Stir. Add 5 c.c. of concentrated nitric acid (*d*); mix, and let stand for five minutes.

3. Add 0.3 gm. ferric ammonium sulphate (*c*).

4. Titrate excess of silver nitrate with the standard potassium sulphocyanate solution (*b*) until the definite salmon-red color of the ferric sulphocyanate persists in spite of stirring for at least fifteen seconds.

Calculation:

Five − number c.c. of (*b*) used in titration × 100 = mg. of chlorid for each 100 c.c. of plasma. To convert chlorid figures in NaCl figures divide by 0.606, or multiply by 1.65.

C. Blood Sugar

In health the blood contains about 100 mg. of sugar, chiefly dextrose, in each 100 c.c., 90 and 120 mg. being widely accepted as the lower and upper normal limits, respectively. Higher values constitute *hyperglycemia*. These figures are based upon blood taken in the morning before breakfast. For a short time following meals rich in carbohydrates there is a slight rise, "normal alimentary hyperglycemia."

Under ordinary conditions the kidneys allow only negligible traces of sugar—too small for detection by the ordinary clinical tests—to pass out into the urine. When, however, from any cause the blood-sugar progressively rises, a point is finally reached when the kidneys no longer hold the sugar back, but begin to excrete it actively, and it then appears in the urine in notable quantities. The point at which the "renal barrier" is overcome is called the "renal threshold for sugar," although the nature and significance of the phenomenon are not well understood. The threshold varies considerably in different persons and under various conditions—is lower, for example, when diuresis exists—but is ordinarily about 170 to 180 mg. blood sugar for each 100 c.c. of blood; it may be

as low as 140 mg. in apparently healthy individuals. Obviously, then, the appearance of sugar in the urine in any given case is the resultant of two factors: (*a*) the blood-sugar concentration and (*b*) the renal threshold.

Ingestion of approximately 100 grams of glucose causes a sharp rise of blood-sugar to about 150, or even 160 mg., in each 100 c.c. in one-half to one hour, the exact time depending somewhat upon the rate of absorption, with a gradual return nearly or quite to the original level by the end of the second hour. Sugar does not appear in the urine. A higher rise or a slower return to the normal, sometimes with the appearance of sugar in the urine, occurs in a number of pathologic conditions to be mentioned later. This is known as the "sugar tolerance test," but the name is somewhat confusing, since low tolerance results in a high blood-sugar curve and high tolerance in a low flat curve. The rate of absorption influences the height of the curve and the time at which this is reached, but does not usually materially affect the type of curve.

The **sugar tolerance test** is carried out as follows[1]:

1. Direct the patient to take no food after 7 P. M.

2. On the following morning obtain blood for determination of the fasting blood-sugar, and give the patient 1.75 gm. dextrose in solution for each kilogram (2.2 pounds) of his body weight. Many workers give 100 gm. of dextrose to all patients. Some patients find it impossible to retain so much. The solution is best taken cold. It is prepared by dissolving pure dextrose in water in the proportion of 10 c.c. of water for each 4 gm. of dextrose and adding the juice of a lemon for flavoring.

3. Secure blood for sugar determinations at the end of the first half-hour, and the first, second, and third hour. In routine work it will generally suffice to take blood at the end of the second and third hours only. It may be possible to enter the vein several times through the same puncture. Estimate blood-sugar and plot a curve.

4. Collect a twenty-four-hour sample of urine, beginning at the time the glucose is given, and make qualitative and quantitative tests for glucose.

The study of blood-sugar has clinical value in diabetes mellitus, in renal glycosuria, and in certain other conditions.

[1] Janney, N. W., and Isaacson, V. I.: A Blood-sugar Tolerance Test, Jour. Amer. Med. Assoc., vol. 70, p. 1131, April 20, 1918.

1. **Diabetes Mellitus.**—The important consideration here is not so much the glycosuria as the hyperglycemia, which the glycosuria is an attempt to relieve. The degree of hyperglycemia depends upon the severity of the disease, upon the diet, and upon the renal threshold. In mild cases blood-sugar may be 150 to 250 mg. in each 100 c.c., and with the lower figure the urine is free from sugar; in severe cases, 300 to 400 mg. or higher. The renal threshold is inconstant: in early cases generally unchanged; in long-continued cases sometimes low, sometimes high. It is particularly high in

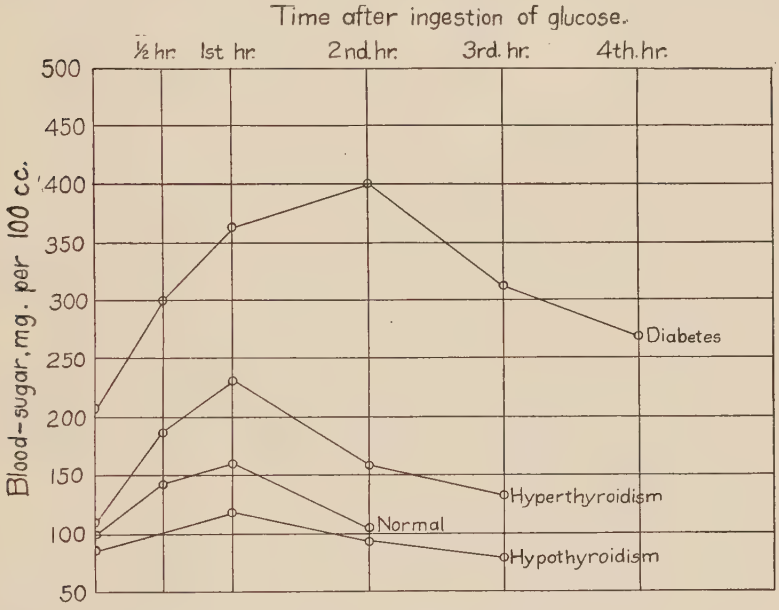


Fig. 162.—Chart showing blood-sugar curves obtained in the tolerance test.

cases with coexisting nephritis, in which sugar may fail to appear in the urine when blood-sugar rises to 300 mg. or above.

In the tolerance test the maximum blood-sugar concentration is generally reached in about two hours, and it often does not fall to the normal level before the end of the third or fourth hour. The test has great value in the diagnosis of very early diabetes and even of the "prediabetic state." Here curves similar to that given in Figure 162 for hyperthyroidism, although often lower, may be expected.

2. **Renal Glycosuria.**—This has been discussed on page 217.

The essential feature is the occurrence of sugar in the urine without increase of sugar in the blood, owing to lowered renal threshold.

3. **Other Conditions with Altered Blood-sugar.**—In general it appears that overfunction of the ductless glands is accompanied by hyperglycemia and underfunction by hypoglycemia, but the change in fasting blood-sugar is so uncertain as to lack diagnostic value. Of much greater significance is the blood-sugar curve after ingestion of glucose. Most conditions of hyperfunction of these glands, particularly hyperthyroidism and hyperpituitarism, cause an accentuation of the curve with more or less pronounced prolongation. The curve is generally much flattened in hypothyroidism, hypopituitarism and Addison's disease. Typical curves are shown in Figure 162.

In chronic nephritis the renal threshold for sugar is generally raised, although sometimes lowered, and the alimentary hyperglycemia curve is generally accentuated and prolonged. Slight or moderate alterations in blood-sugar occur in many other diseases, but are probably of no diagnostic importance. A diminished sugar tolerance may be due to diminished ability of the liver to store glycogen.

The methods described below are those that have been found useful in most laboratories. Recently, however, both Folin[1] and Benedict[2] have devised new methods which may in time replace their older methods.

Method of Folin and Wu.—*Reagents Required.*—(*a*) Standard sugar solutions. Prepare a stock solution containing 10 gm. pure anhydrous dextrose in 1000 c.c. distilled water, and preserve with a little xylene. From this prepare the two standard solutions used for blood-sugar determinations as follows:

Standard No. 1. Place 5 c.c. of the stock solution in a 500-c.c. volumetric flask, add distilled water to the mark, and mix. Two c.c. of this standard solution contain 0.2 mg. dextrose.

Standard No. 2. Place 5 c.c. of the stock solution in a 250-c.c. volumetric flask, add distilled water to the mark, and mix. Two c.c. of this standard solution contain 0.4 mg. dextrose.

[1] Folin, Otto: The Determination of Sugar in Blood and Normal Urine, Jour. Biol. Chem., vol. 67, p. 357, February, 1926.

[2] Benedict, S. R.: The Estimation of Sugar in Blood and Normal Urine, Jour. Biol. Chem., vol. 68, p. 759, June, 1926.

(*b*) Alkaline copper sulphate solution. Dissolve 40 gm. pure anhydrous sodium carbonate in about 400 c.c. of distilled water, and place in a 1000 c.c. volumetric flask. In this dissolve first 7.5 gm. of tartaric acid, and then 4.5 gm. crystalline copper sulphate. Make up to 1000 c.c. with distilled water, and mix. Should a precipitate of cuprous oxid form, after a week or two, owing to use of impure chemicals, it may be removed by filtering through a good quality paper.

(*c*) Molybdate phosphate solution. In a large beaker place 35 gm. of molybdic acid, 5 gm. of sodium tungstate, 200 c.c. of 10 per cent. sodium hydroxid, and 200 c.c. of water. Boil vigorously for twenty to forty minutes. Cool, dilute to about 350 c.c., and add 125 c.c. of 85 per cent. phosphoric acid. Make up to 500 c.c. with distilled water, and mix.

Method.—1. Transfer 2 c.c. of the protein-free blood filtrate, representing 0.2 c.c. blood (p. 334), to a Folin blood-sugar tube (Fig. 163).

Into a second blood-sugar tube place 2 c.c. of standard sugar solution No. 1, containing 0.2 mg. dextrose.

Into a third tube place 2 c.c. of standard sugar solution No. 2, containing 0.4 mg. dextrose.

2. To each tube add 2 c.c. of the alkaline copper sulphate solution, and mix. The surface of the fluid must reach the constricted portion of the tubes, but must not go above it.

3. Immerse the tubes in boiling water for six minutes.

4. Place in cold water, without shaking, for two to three minutes.

5. To each tube add 2 c.c. of the molybdate phosphate solution.

Fig. 163.—Folin's tube for use in blood-sugar estimations. The narrow portion is 4 cm. long and 8 mm. in diameter. The bulb must be of such size that 4 c.c. of fluid will reach into the lower third of the constricted portion, and not above it.

6. When the precipitate of cuprous oxid has dissolved, which should be within two minutes, dilute with water to the 25 c.c. mark, insert a rubber stopper, and mix thoroughly.

7. Compare the unknown in a colorimeter with the standard which it more nearly matches. Calculate the amount of sugar in 100 c.c. of blood, upon the basis that the unknown represents 0.2 c.c. of blood and the two standards 0.2 and 0.4 mg. of dextrose, respectively. With the Duboscq type of colorimeter the following formula is applicable, D representing the number of milligrams of dextrose in the standard:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times D \times 500 = \text{mg. sugar in 100 c.c. of blood.}$$

Lewis and Benedict Method (*Myers and Bailey Modification*).—*Reagents Required.*—(*a*) Picric acid, chemically pure. For each determination 0.5 gm. will be required.

(*b*) Sodium carbonate, saturated solution (22 per cent.). Each determination requires 2 or 3 c.c.

(*c*) Standard dextrose solution, a 0.02 per cent. solution of pure dextrose in saturated aqueous solution of picric acid.

Method.—1. Place 2 c.c. of oxalated blood in a centrifuge tube, preferably with round bottom to facilitate stirring; and add 8 c.c. of distilled water.

2. Mix well, and let stand until the blood is completely laked.

3. Add 0.5 gm. of dry picric acid and stir well with a slender glass rod. Let stand for five minutes, with occasional stirring.

4. Centrifugalize, and filter the supernatant fluid through a small filter-paper into a dry test-tube.

5. Transfer 3 c.c. of the filtrate to a 10-c.c. volumetric flask (a tall test-tube with a 10-c.c. mark or an accurately graduated centrifuge tube will answer). Add 1 c.c. saturated solution of sodium carbonate.

6. Place 3 c.c. of the standard dextrose-picric-acid solution in a similar flask or tube, and add 1 c.c. of saturated sodium carbonate solution.

7. Heat both tubes in a beaker of boiling water for fifteen to twenty minutes. Cool to room temperature.

8. Make up the unknown and the standard solution to 10 c.c. with distilled water. Mix well.

9. Compare the unknown with the standard in a colorimeter. The calculation is based upon the fact that the unknown represents 0.6 c.c. of blood and the color standard 0.6 mg. of dextrose. With the plunger type of colorimeter or the Denison Laboratory colorimeter the following formula may be used:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 100 = \text{mg. of dextrose in 100 c.c. of blood.}$$

When the blood-sugar is high a standard containing double the amount of dextrose-picric-acid solution must be used, and this requires that the final result be multiplied by 2.

D. Carbon Dioxid Combining Power of Blood-plasma

As is shown in the section upon Acidosis, page 563, the determination of the capacity of the blood-plasma to bind carbon dioxid offers what is probably the most reliable means of detecting acidosis and of measuring its degree. The normal range for adults is 53 to

75 c.c. of carbon dioxid in each 100 c.c. of blood plasma; infants, 40 to 55 per cent. Figures below 50 per cent. in adults indicate acidosis; below 30 per cent., severe acidosis. Determinations are especially useful as a guide in alkali treatment of acidosis, during which the carbon dioxid combining power steadily rises. Palmer and Van Slyke estimate that as a rule 0.5 gram of sodium bicarbonate for each 42 pounds of body weight will raise the plasma carbon dioxid volume 1 per cent.

Method of Van Slyke and Cullen.—I. *Obtaining the Blood.*—The patient should avoid muscular exercise for an hour or two before the blood is taken. About 6 to 8 c.c. of blood are required. Withdraw this from a vein with no more constriction than is necessary, and with the least possible exposure to the air; and immediately place it in a tube

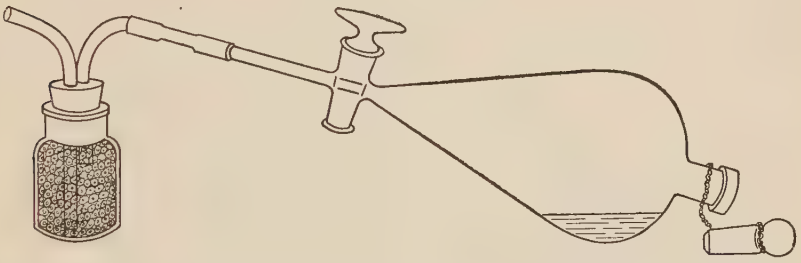


Fig. 164.—Apparatus used for saturating blood plasma with carbon dioxid. Air is blown from the lungs into the tube at the left, the stopper is inserted and the separatory funnel rotated. The air passes among the glass beads in the bottle which remove its moisture. (After Van Slyke.)

with enough dry potassium oxalate to make about 0.5 per cent. Mix by gentle stirring. Separate the plasma by thoroughly centrifugalizing within half an hour.

A better plan is to secure the blood under oil in a device similar to that shown in Figure 99, but with the entrance tube reaching to the bottom in which has been placed 2 or 3 c.c. of paraffin oil and the dry potassium oxalate. When this plan is followed it is essential that the arm constriction be loosened before the blood is withdrawn.

II. *Saturation with Carbon Dioxid.*—1. Transfer about 3 c.c. of the plasma to a large separation funnel arranged as shown in Figure 164.

2. Without inspiring more deeply than is normal, blow air from the lungs into the funnel through the bottle of beads, which is used to remove excessive moisture from the air. Expiration should be rather rapid and as complete as possible. Insert the stopper just before expiration is finished and close the stop-cock.

3. Rotate the funnel for two minutes, during which time the plasma should be distributed as completely as possible over the inner surface of the funnel. Carry out the analysis without delay.

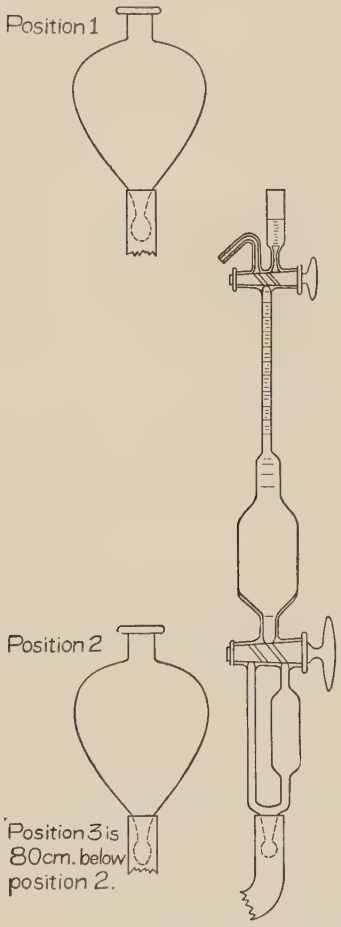


FIG. 165.—Van Slyke's apparatus for carbon dioxid of blood, showing the three positions of the mercury bulb. The bulb is connected to the apparatus by a heavy rubber tube which is merely indicated in the drawing. The apparatus is set up on an iron ring stand.

III. *The Analysis.*—A special apparatus devised by Van Slyke is required (Fig. 165). The directions appear complicated, but the manipulation of the apparatus is not difficult.

1. Set up the apparatus upon a ring stand with mercury bulb held at position 1. The instrument must be firmly held by a clamp whose jaws are protected by heavy rubber and a support should be placed below the lower stop-cock. Completely fill the apparatus with mercury by pouring it into the bulb. About 3 pounds will be required. Make sure that both openings in the lower and upper stop-cocks are filled, and also the two capillaries above the upper stop-cock. The stop-cocks should be well anointed with vaselin, and should be held securely in place by means of heavy rubber bands.

2. With the upper stop-cock closed and the lower one open, make sure that there is no leak by lowering the mercury bulb to position 2. This should produce a vacuum in the upper part of the apparatus; and when the bulb is returned to position 1, the mercury should fill it completely and strike the upper stop-cock with a click. If air has entered, it forms a cushion and the click is not heard.

3. Rinse out the cup at the top of the apparatus with distilled water. Leave 1 c.c. of the water in the cup.

4. Transfer to the cup 1 c.c. of the plasma which has been freshly saturated with carbon dioxid, keeping the tip of the pipet below the surface of the fluid. Add 1 small drop of pure caprylic alcohol.

5. Place the mercury bulb in position 2, and by carefully turning

the upper stop-cock allow the plasma and water to flow down into the main stem of the apparatus, leaving the capillary portion of the cup filled with the fluid.

6. Place 0.5 c.c. of 10 per cent. lactic acid in the cup and admit enough of the acid into the main stem of the apparatus to bring the level of the fluid down exactly to the 2.5 mark.

7. Place a drop of mercury in the cup and let it run down in the narrow portion to seal the stop-cock.

8. Lower the mercury bulb to a position about 80 cm. below the lower stop-cock, where it may be supported by a ring or hook on the side of the work-table.

9. When the level of the mercury in the apparatus has fallen to the 50 c.c. mark close the lower stop-cock.

10. Remove the apparatus from its clamp, without disconnecting the rubber tubing, and turn it upside down at least fifteen times in order to thoroughly agitate the contents.

11. Replace the apparatus in its clamp, and turn the lower stop-cock so that the fluid is drained as completely as possible into the bulb beneath. To avoid letting any of the gas escape with the fluid the last drop or two of fluid may be allowed to remain.

12. Raise the levelling bulb very slowly with the left hand, and with the right turn the lower stop-cock so that the mercuty rises gradually into the apparatus through the left-hand tube below the stop-cock. The mercury will fill the body of the apparatus and as much of the graduated stem at the top as is not occupied by the carbon dioxid liberated from the blood-plasma. Leave the lower stop-cock open.

13. Now place the levelling bulb at such height that the surface of the mercury is at exactly the same level as that in the apparatus.

14. At once read off the volume of gas in the graduated stem. This, when corrected for temperature, barometric pressure, and other factors, represents the carbon dioxid bound by 1 c.c. of blood-plasma. For clinical work the following method will suffice if the room temperature is near 18° or 20° C. Find the barometric pressure in millimeters, divide by 760, multiply by the gas-volume reading on the apparatus, and subtract 0.12. The result, multiplied by 100, gives the amount of carbon dioxid bound by 100 c.c. of plasma, or the "volume per cent." *Example:* Suppose the volume of gas in the graduated stem of the apparatus was 0.6 c.c., and suppose that the barometric pressure at the time was 754 mm.

$754 \div 760 = 0.992$

$0.6 \times 0.992 = 0.5952$ = reading corrected for pressure.

$0.5952 - 0.12 = 0.4752$ = fully corrected volume CO_2 in 1 c.c. plasma.

$0.4752 \times 100 = 47.52$ = volume CO_2 in 100 c.c. plasma, "volume per cent."

15. When not in use the apparatus should be filled with water. Clean the mercury occasionally by filtering through chamois skin.

E. Calcium; Phosphorus

In parathyroid tetany, the calcium content of the blood-serum falls below the normal amount of 9 and 11 mg. for each 100 c.c. The condition is relieved by administering calcium. Inorganic phosphorus is reduced below the low limits of normal (3.7 mg. for each 100 c.c.) in rickets, whereas in adults with major fractures the level rises above normal. This level may remain high if the fracture is ununited. The student is referred in the footnote[1] to satisfactory methods for determining calcium and phosphorus.

F. Bile-pigment

The faint yellowish tinge of normal blood-plasma and blood-serum is probably due to minute traces of bilirubin. In jaundice the yellow color becomes much deeper, and this may be evident in hepatic and hemolytic diseases at a time when no jaundice of the skin or sclera can be discerned. The recognition of bile-staining of the plasma is, therefore, a very sensitive and very definite means of detecting slight grades of jaundice and of recognizing fluctuations in the severer grades. Clinically the "icterus index" is used as a means of distinguishing hemolytic from non-hemolytic anemias, as a test of liver function, and as a means of recognizing very early biliary obstruction.

No other yellow pigment is present in the blood in appreciable quantities, excepting occasionally, that of carrots. A diet of carrots, especially popular for young children, may give a peculiar yellow tinge to the skin and blood-serum, known as carotinemia. It does no harm and disappears after removing the vegetable from the diet.

In testing for bile-pigment either plasma or serum may be used, but the blood must be secured with careful avoidance of hemolysis. Serum is obtained in the usual way (p. 610). For plasma the blood is secured in a tube or syringe containing a trace of powdered potassium oxalate to prevent clotting, and is then centrifugalized.

[1] Clark, E. P., and Collip, J. B.: A Study of the Tisdall Method for the Determination of Blood-serum Calcium with a Suggested Modification, Jour. Biol. Chem., vol. 63, p. 461, March, 1925. Fiske, C. H., and Subbarow, Y.: The Colorimetric Determination of Phosphorus, Jour. Biol. Chem., vol. 66, p. 375, December, 1925.

Test for Bile Pigment.—Gmelin's test may be applied by overlaying the yellow nitric acid with the serum or plasma. A broad white ring of coagulated protein appears at the zone of contact. The appearance of a narrow bluish-green ring in the midst of the white indicates an abnormal amount of bile-pigment. The serum of normal persons will sometimes give a very faintly positive reaction, the colored ring becoming evident only after half an hour. The test is said to detect 1 part of bilirubin in 30,000 or 40,000 of serum.

Blankenhorn's Method.—This is a simple means of roughly measuring the degree of jaundice. The plasma or serum, which must show no trace of hemoglobin, is diluted with distilled water until its yellow color is just perceptible when viewed in a layer 1 cm. thick; and the degree to which it is diluted is taken as a measure of the bilirubin content. A white cloud of precipitated globulin often appears. This may be dissolved by adding a drop of ammonia.

Dilution is best carried out in a cylinder graduate. The end-point is determined by comparing a 1 c.c. column of the diluted serum in a test-tube (best with a flat bottom) with a similar column of distilled water in another tube. The ends of the tubes should be immersed in about an inch of water in a large porcelain evaporating dish and the column viewed from above.

The number of dilutions required to reduce the color to a barely perceptible yellow is recorded as the index of bilirubin. If, for example, 0.5 c.c. of plasma were used and the total volume after dilution were 80 c.c., then the result is recorded as 160 dilutions. Values under 10 and, probably, 15 dilutions should not be considered abnormal. A plasma with dilution value of 20 will generally give a definite Gmelin's test.

More recently Bernhard and Maue, modifying Meulengracht's method, have made use of the Duboscq colorimeter in which the blood-serum, diluted 1 : 2, 1 : 4, 1 : 10, and so forth, as may be necessary, is compared with a color-standard consisting of 1 : 10,000 solution of potassium bichromate. The reading of the standard is divided by the reading of the unknown and this multiplied by the dilution to obtain the "icterus index." Stetten has found the normal by this method to lie between 2.5 and 5, average 3.6.

Van den Bergh Quantitative Method for Serum Bilirubin. (*Modification of Thannhauser and Anderson*[1]).—Ehrlich found that when sulphanilic acid and sodium nitrite were added to a solution of bilirubin,

[1] Thannhauser, J. S., and Anderson, E.: Methodik der quantitativen Bilirubin Bestimmung im menschlichen Serum, Deutsch. Arch. f. klin. Med., vol. 137, p. 179, August, 1921; abst. Jour. Amer. Med. Assoc., vol. 77, 1292, October, 1921. Greene, C. H., Snell, A. M., and Walters, Waltman: Diseases of the Liver. I. A Survey of Tests for Hepatic Function, Arch. Int. Med., vol. 36, p. 248, September, 1925.

a colored addition product, azobilirubin was formed. This reaction is specific and will detect bilirubin in a dilution of 1 : 1,500,000. Van den Bergh precipitated the blood-serum protein with alcohol, which also serves as a solvent for bilirubin, and showed that Ehrlich's reaction could be applied to this alcoholic extract. When a very small amount of bilirubin is present in the blood-serum, with hemolysis as the source of the pigment, alcoholic extraction is necessary. This is the principle of the "indirect reaction." On the other hand, in obstructive jaundice with bile itself in the blood-serum, alcoholic extraction is not necessary, as the characteristic color reaction appears at once on addition of the reagent. This is the "direct reaction." Either serum or plasma may be used for the test, but it must be clear and free from hemoglobin.

Reagents Required.—(*a*) Standard artificial bilirubin solution. (1) Dissolve 0.1508 gm. of ammonium iron alum in 50 c.c. of concentrated hydrochloric acid, and add water to make 100 c.c. This solution will keep indefinitely.

(2) To 10 c.c. of (1) add 25 c.c. concentrated hydrochloric acid, and water up to 250 c.c. This solution keeps about six months.

(3) To 5 c.c. of (2) add 5 c.c. of 20 per cent. potassium sulphocyanid in a glass stoppered cylinder (50 c.c. capacity); add 20 c.c. of ether. Shake well and transfer ether extract to a colorimeter cup. This standard solution must be prepared each day a test is made.

(*b*) Sulphanilic reagent. This is freshly prepared Ehrlich's diazo reagent. It is made of two solutions each of which keeps well, but the mixture must be made immediately prior to the test.

Solution A: Sulphanilic acid, 5 gm.; concentrated hydrochloric acid, 50 c.c.; distilled water to make 1000 c.c.

Solution B: sodium nitrite, 0.5 gm.; distilled water, 100 c.c.

Take 0.8 c.c. of solution B and make up to 25 c.c. with solution A.

(*c*) Saturated solution of ammonium sulphate.

(*d*) Ethyl alcohol, 95 per cent.

Method.—1. To 2 c.c. of clear serum add 1 c.c. of freshly prepared sulphanilic reagent (*b*). If there is a marked color change this may be compared at once with the standard; the "direct reaction."

2. Add 2 c.c. saturated ammonium sulphate (*c*).

3. Add 10 c.c. 95 per cent. alcohol (*d*).

4. Centrifugalize.

5. Compare with standard (*a*) in a Duboscq colorimeter, setting standard cup at 20 mm.

Calculation:

$$\frac{\text{Reading of Standard (20)}}{\text{Reading of Unkown}} \times 3.75 = \text{mg. of bilirubin in 100 c.c. of serum.}$$

Normal blood contains less than 2 mg. of bilirubin for each 100 c.c. of serum.

G. Bromsulphalein Test of Liver Function

Following the great success of the phenolsulphonephthalein test of kidney function, Rowntree and his co-workers applied a similar test to the liver, using phenoltetrachlorphthalein, which they found to be excreted practically only by the liver and to be non-toxic. A definite dose was administered intravenously and the amount recoverable from the feces in forty-eight hours was taken as an index of the functional capacity of the liver. For various reasons the test did not meet general favor. It was later modified by H. L. McNeil, who determined the time required for the dye to appear in the bile and measured the amount which could be recovered from the duodenum within two hours by means of the duodenal tube, but obtained inconstant results. In 1922 Rosenthal introduced the method of measuring the amount which remains in the blood at definite periods after intravenous injection of 5 mg. of dye per kilogram of body weight. More recently he substituted bromsulphalein for phenoltetrachlorphthalein. He has found that a normal liver will remove most of the dye from the blood within fifteen minutes and practically all of it within an hour; while in animals whose livers have been damaged experimentally and in human beings with definite hepatic disease the dye is removed much more slowly, and considerable amounts may remain for three hours or longer.

Rosenthal's Bromsulphalein Method.[1]—The dye is conveniently used in 5 per cent. solution. One-tenth cubic centimeter then contains 5 mg. of the dye. This solution, sterilized and put up in sealed ampules, may be obtained of Hynson, Westcott, and Dunning, Baltimore.

Method.—1. Weigh the patient, calculate the amount of dye required for the test, allowing 5 mg. for each kilogram of body weight. For each 10 kilograms of body weight take 1 c.c. of the sterile dye solution as furnished by the manufacturer.

2. Draw the required amount of dye into an accurately calibrated glass syringe, and inject the dye, without diluting, into a vein in the patient's arm. Take one minute for the injection.

3. At the end of thirty minutes and again one hour after the injection,

[1] Rosenthal, S. M., and White, E. C.: Clinical Application of the Bromsulphalein Test for Hepatic Function, Jour. Amer. Med. Assoc., vol. 84, p. 1112, April, 1925.

secure 5 c.c. of blood from the other arm, using a dry needle to avoid hemolysis. Collect the blood in a dry test-tube, allow it to clot, and centrifugalize to separate the serum.

4. Determine the amount of dye in the thirty-minute and one-hour samples of blood. This determination is best made in the special colorimeter furnished by the manufacturer (Fig. 166). Equal quantities of the serum from each specimen are pipetted off into three small test-tubes of the same diameter as the color tubes of the colorimeter. To one of the tubes add a drop of 10 per cent. sodium hydroxid. To the others may be added a drop of 5 per cent. hydrochloric acid if the serum is colored with hemoglobin. Place the tubes in the colorimeter side by side and match by placing the standard tubes behind the tubes without the alkali. The percentage strength of the dye is marked on the standard tube.

FIG. 166.—Rosenthal colorimeter for bromsulphalein liver function test. Supplied by Hynson, Westcott, and Dunning, Baltimore, Md.

Grading of the degree of impairment of liver function is made chiefly on the amount of dye retained in the one-hour specimen:

Grade.	Percentage dye retained.
0 (normal)	Less than 15
1	Less than 30
2	Less than 60
3	Less than 100
4	More than 100

XIV. MISCELLANEOUS METHODS

1. Vital Staining.—(1) **Red Corpuscles.**—Upon the assumption that ordinary staining of dried and fixed blood-films gives the reactions of dead cells and does not necessarily indicate the condition of the living blood, attempts have been made to stain blood-cells in the living state. The information yielded by this so-called vital or "supravital" staining has to do chiefly with certain "reticulated" red corpuscles which contain a coarse skein or network of granular filaments. Sometimes, apparently depending upon varia-

tions in technic of staining, there are no definite filaments, but only a number of discrete coarse granules scattered irregularly through the cell. The granulofilamentous substance stains sharply with many of the basic dyes when these are applied to the living cells, but completely fails to stain in the usual dried films.

As to the nature of the reticulum little is known definitely. Some workers connect it with polychromatophilia, others with mitochondria. In any case reticulation is apparently a characteristic of very young red corpuscles, and the number of these in the circulating blood is probably the best available index of the activity of blood regeneration. An increase may be interpreted as indicating both an excessive demand for new cells and a competent bone-marrow; when the demand lessens or the bone-marrow fails, the number of reticulated red cells falls.

In healthy medical students we have found the reticulated cells to range from 0.3 to 1 per cent. of all the red corpuscles, usually 0.6 to 0.8 per cent. In young infants the percentage is two to four times as high. Some writers give higher figures: 0.5 to 2 per cent. for adults, 5 to 10 per cent. for infants. In the various anemias associated with increased activity of the bone-marrow they range from 2 to 20 per cent. or even higher, the percentage running parallel with the varying activity of blood regeneration and bearing no necessary relation to the red cell count. The percentage is notably high in hemolytic jaundice.

Of the various dyes which have been used for vital staining, brilliant cresyl blue appears to be by far the most satisfactory. Most of our work has been done with Grübler's dye, but at least one dye of American make has proved equally good. We are now using the following method:

1. In a centrifuge-tube with conical tip place the following solutions, and mix:

1 per cent. brilliant cresyl blue in 0.85 per cent. sodium chlorid solution, freshly filtered	5 drops
.1 per cent. neutral potassium oxalate in 0.85 per cent. sodium chlorid solution	25 "

2. Prick the finger or ear deeply and allow 2 or 3 drops of blood to fall into the stain. Mix gently and let stand ten to twenty minutes.

3. Centrifugalize for a few moments and by means of a capillary pipet remove most of the supernatant fluid, leaving a volume equal to

the volume of corpuscles. Mix the sediment with the remaining fluid, transfer a small drop to a clean slide, spread in the usual way, and dry in the air.

The sediment must be sufficiently concentrated to allow of quick drying in order to avoid distortion of the corpuscles and to give films with the red cells lying close together but not overlapping. Also the sediment must be well-mixed to prevent unequal distribution of the reticulated cells owing to a possible difference in their specific gravity.

4. Examine with an oil-immersion lens and calculate what proportion of the red corpuscles show reticulum or granules. As a basis for calculation at least 1000 cells (better 3000) should be examined for reticulation, and these on several different portions of the slide. To obviate the difficulty of counting large fields with a confusing number of cells, the field of view may be divided by cementing four short pieces of a hair

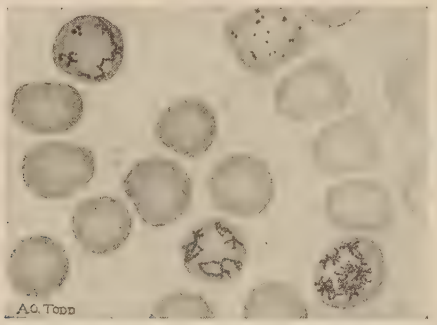


FIG. 167.—Reticulated red corpuscles, drawn from two slides stained as described in the text. The red corpuscles were yellowish green. the reticulum and granules, blue (× 1000).

across the diaphragm of the ocular in such a way as to form a small rectangle in the center.

In preparations made as ab ve described the leukocytes and platelets are colored shades of blue and the red corpuscles yellowish-green. The reticulated corpuscles are often decidedly larger than their fellows. The network and granules are deep blue and stand out distinctly (Fig. 167). The only serious source of error will be particles of stain adhering to the surface of the corpuscles. The preparations retain their color well if kept from the light.

When desired the films may also be stained with Wright's stain which, combined with the brilliant cresyl blue, gives beautiful preparations.

(2) **Leukocytes.**—Florence Sabin has introduced a simple method of studying the leukocytes in the living condition which

opens a new and fertile field for research, and also promises to become a useful clinical procedure, although sufficient facts have not yet been accumulated to give it clinical value at present. For details the reader is referred to the papers of Sabin and her co-workers.[1]

A similar method has long been in use for vital staining of red corpuscles, but the concentration of dye which stained the red cells satisfactorily injured the white. Moreover, in the case of the red cells it was not necessary to keep the cells alive during the examination.

Technic.—1. Carefully clean slides and covers as follows: Soak in bichromate cleaning fluid three or four days; place in running water two or three hours, separating slides occasionally; rinse in three or four changes of distilled water, and soak over night; store in 80 per cent. alcohol. Before use wipe with a clean towel and polish slides with jeweler's rouge applied on a piece of silk.

2. Flame the slides and flood with a very dilute solution of the selected dye in absolute alcohol, drain off excess, and place the slides upright until dry. The film must be very thin and evenly spread. Prepared slides may be stored until needed, but must be kept from dust. Many stains will serve. Sabin prefers neutral red (specify for vital staining) as it is relatively non-toxic and is also an indicator, showing the chemical reaction of the parts of the cell which take the stain. The very dilute solution with which the slides are spread is prepared by mixing 0.4 c.c. of a 1 per cent. solution of neutral red in absolute alcohol with 10 c.c. absolute alcohol. When it is desired also to bring out the mitrochondria of the cells, 3 drops of saturated solution of Janus-green in absolute alcohol may be added to each 2 c.c. of the diluted solution of neutral red.

3. Receive a very small drop of blood from a puncture on a perfectly clean cover, invert upon the prepared slide, and immediately seal with vaselin of high melting point or with a mixture of vaselin and paraffin. The slide must not be cold, but need not be warmed.

[1] Sabin, Florence R.: Studies of Living Human Blood-cells, Bull. Johns Hopkins Hosp., vol. 34, p. 277, September, 1923. Sabin, Florence R., Austrian, C. R., Cunningham, R. S., and Doan, C. A.: Studies on the Maturation of Myeloblasts into Myelocytes. Jour. Exp. Med., vol. 40, p. 845, December, 1924. Sabin, Florence R., Cunningham, R. S., Doan, C. A., and Kindwall, J. A.: The Normal Rhythm of the White Blood-cells, Bull. Johns Hopkins Hosp., vol. 37, p. 14, July, 1925. Cunningham, R. S., Sabin, Florence R., Sagiyama, S., and Kindwall, J. A.: The Rôle of the Monocyte in Tuberculosis, Bull. Johns Hopkins Hosp., vol. 37, p. 231, October, 1925.

4. Within ten minutes place the slide on a warm stage or better, in a microslide incubator. Examine at once with the oil-immersion objective. Normally the cells remain alive for at least an hour, sometimes three or four hours.

Each lot of cleaned slides and each new bottle of diluted stain should be tried out with normal blood to insure uniform conditions. Leukocytes are very sensitive to the least trace of acid that may be left on the slides and to an excess of the dye. Any coloring of the nucleus indicates injury to the cell.

Appearance of Leukocytes.—*Polymorphonuclear neutrophilic leukocytes* are constantly moving about with their characteristic ameboid motion and are readily seen. Their cytoplasmic granules, which are numerous, of small size and pale red, are constantly streaming through the cytoplasm. As the cell moves about, the nucleus with its several lobes, is usually in the rear part. In addition to the granules, the cytoplasm of most of these cells contains one or more rounded bodies which take the stain slowly. These are larger than the granules and are presumably digestive vacuoles, indicating phagocytic activity. They vary in color, depending on the chemical reaction of the contents. Certain leukocytes are rounded and motionless, the nucleus is structureless and nearly fills the cell, and the granules, although distinctly visible, do not take the stain. These are interpreted as dead or dying neutrophils. Their number varies at different hours, being usually greatest near the middle of the day. The number may be greatly increased by faulty technic (excessive heat, pressure of the cover, and so forth).

The cytoplasmic granules of *eosinophils* and *basophils* stain with the neutral red. The basophilic granules are somewhat smaller than the eosinophilic, and differ among themselves in size and depth of staining. Digestive vacuoles are apparently absent from both cells. Eosinophils are actively motile, basophils slightly so.

The younger *monocytes*, with oval nuclei, are rounded and practically non-motile. Their cytoplasm contains very fine salmon-colored granules which are grouped around a clear spot, the centrosphere. With these fine granules are a variable number of larger red bodies, apparently digestive vacuoles. The older cells, with lobulated or saddle-shaped nuclei, are more irregular in shape, usually elongated, and are sluggishly motile. The red vacuoles are usually numerous and may displace or at least obscure the fine granules.

The cytoplasm of *lymphocytes* is clear excepting for a few small vacuoles which take the red stain, and, when Janus-green is added, a clump of blue mitrochondria opposite the nucleus. The nucleus is oval or indented, seldom round. The large and small lymphocytes show no

locomotion. Those of intermediate size move very slowly, and the nucleus is then at the front end and changes its shape.

3. Fragility of Red Corpuscles.—(1) **Resistance to Hypotonic Salt Solution.**—Destruction of red corpuscles goes on continually within the body. In certain pathologic conditions this destruction is greatly accelerated, leading usually to anemia; and it is then of great practical interest to ascertain whether the excessive hemolysis is referable chiefly to increased fragility of the red cells, as is typically the case in hemolytic jaundice, or to an excessively toxic hemolytic agent acting upon red cells of comparatively normal resisting power, as is usual in pernicious anemia. The resistance of the red cells can be measured by subjecting them to the action of various harmful agents. In clinical work hypotonic salt solution is generally used.

Method of Sanford.—1. Prepare a stock solution containing 0.5 gm. chemically pure and freshly dried sodium chlorid in 100 c.c. of distilled water. Weigh on a delicate balance and measure in a volumetric flask.

2. Arrange a series of 12 small test-tubes in a rack and number them 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, and 14.

3. With a capillary pipet place in each tube the number of drops of the stock 0.5 per cent. salt solution indicated by the number on the tube. To insure equality in size of drops the pipet must always be held at the same angle.

4. With the same pipet add to each tube the number of drops of distilled water required to bring the volume in each tube to 25 drops. Mix well. The percentage strength of the salt in any tube may then be found by multiplying its number by 0.02.

5. Obtain 1 or 1.5 c.c. of the patient's blood from a vein with a small dry sterile syringe and No. 21 needle, and immediately expel 1 drop into each of the tubes. Mix by inverting (Fig. 168).

If some time must elapse before the blood can be added, it may be mixed with citrated salt solution (p. 609) and the corpuscles washed with 0.7 per cent. salt solution before use. In this case make a 50 per cent. suspension of the cells and add 1 drop to each of the tubes in the rack.

6. Prepare a similar set of tubes, using the blood of a normal person as a control. *This should not be omitted.* The normal control serves as a test of the accuracy of the salt solution, and at the same time gives a definite standard for the interpretation of slight changes in fragility.

7. Let the tubes stand two hours at room temperature. At the end of that time the corpuscles will have settled to the bottom and hemolysis

may be recognized by the color of the supernatant fluid: faintly pink if hemolysis is partial ("initial hemolysis"); red, with little or no sediment, if it is complete.

With normal blood, hemolysis usually begins in the tube containing 0.44 or 0.42 per cent. salt solution and is complete in that containing 0.34 per cent. When a control is used a variation of 0.02 or 0.04 may be considered quite definite. Sanford found the average figures for initial and complete hemolysis in 23 cases of hemolytic jaundice to be 0.478 and 0.413 respectively; in chronic obstructive jaundice, 0.396 and 0.31. In secondary and pernicious anemia the figures vary only slightly from the normal, with a tendency to slight increase of resistance. In purpura resistance is normal.

Fig. 168.—Set-up for fragility test; adding the blood.

(2) **Resistance of Red Corpuscles to Cobra Venom. Cobra Venom Test for Syphilis.**—Through the work of Weil in 1909 it became known that the red corpuscles of syphilitic individuals are characteristically resistant to hemolysis by cobra venom. This is of an entirely different nature from the resistance to hypotonic salt solution just described. The exact explanation of the phenomenon is not wholly clear. It appears to depend upon the same disturbance of lipoid metabolism which is responsible for the Wassermann reaction.

Materials Required.—1. The *cobra venom* may be obtained from Poulenc Frères, Paris. The stock solution is a 1 : 1000 dilution in physiologic salt solution, made very accurately. It is best preserved in 1 c.c. amounts in sealed ampules, and kept frozen in a vacuum bottle containing salt and ice.

2. *The Blood-cells to be Tested.*—Have ready physiologic salt solution to which 2 per cent. of sodium citrate has been freshly added and which has been cooled in the refrigerator. Place about 8 or 10 cc.. of this in a graduated centrifuge tube, add about 2 c.c. of the patient's blood freshly obtained from a vein, and mix very gently. The red cells should stand in contact with the citrate solution overnight in the refrigerator, or, if necessary, they may be prepared at once. Wash at least three times with physiologic salt solution, running the centrifuge at low speed. The last washing of all bloods in a series should be done at the same speed and for the same length of time. After the last washing note the volume of the cells in the bottom of the tube and add saline to bring the total volume up to twenty-five times this volume. This makes a 4 per cent. cell suspension.

Method.—From the stock solution of venom prepare the following dilutions for the test: 1 : 10,000, 1 : 15,000, 1 : 20,000, 1 : 30,000, 1 : 40,000. Place 6 serologic tubes in a rack. In the first 5 tubes place 1 c.c. of the several venom dilutions and 1 c.c. of the cell suspension. In the sixth tube place 1 c.c. of the 1 : 30,000 dilution and 1 c.c. of a 4 per cent. suspension of red cells of a normal person. Mix gently and incubate for one hour at 37° C. Make a preliminary reading, mix gently, and place in the refrigerator until next morning, when the final reading is made. The two readings should correspond very closely.

Readings are made as indicated in the table below, which follows Kolmer. H means complete hemolysis; PH, partial hemolysis; N, complete lack of hemolysis. The control tube No. 6 should show complete hemolysis.

Tube 1.	Tube 2.	Tube 3.	Tube 4.	Tube 5.	
H	PH	N	N	N	= strongly positive
H	H	PH	N	N	= moderately positive
H	H	H	PH	N	= weakly positive
H	H	H	H	PH	= negative
H	H	H	H	H	= certain negative

The cobra venom reaction appears later in syphilis than does the Wassermann reaction, but yields a distinctly higher percentage of positives in tertiary and latent syphilis. Furthermore, it may react positively in treated cases for some time after the Wassermann test has become negative. Upon the other hand, it yields only about 20 per cent. of positives in tabes and paresis, and is negative in primary syphilis. Positive reactions sometimes occur in cancer, but otherwise they are limited to syphilis.

3. Viscosity.—It is evident that variations in the viscosity of

the blood must markedly influence the load carried by the heart, but viscosity estimations have proved of comparatively little value. The greatest field would seem to be in suggesting need for treatment when high viscosity is throwing an excessive burden upon an already weakened heart.

Compared with distilled water, the normal viscosity is about 4.5. It is reduced in primary and secondary anemia (roughly proportional to the grade of anemia), nephritis, cardiac lesions with edema, and usually in leukemia and malaria. It is increased in polycythemia, diabetes mellitus, icterus, and usually in pneumonia.

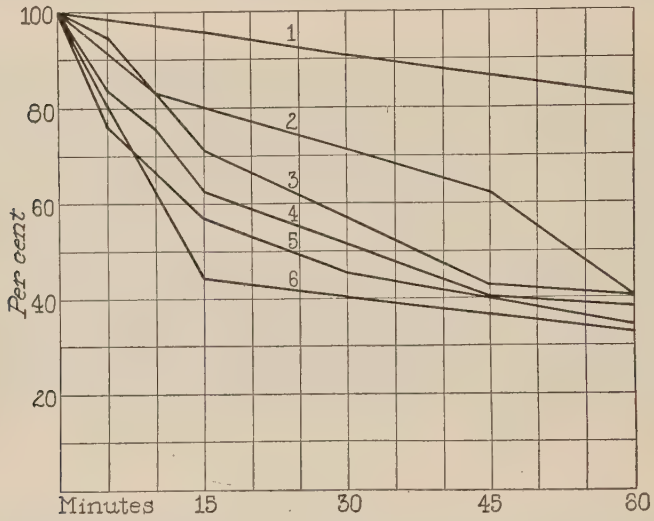


FIG. 169.—Rates of sedimentation of red corpuscles in various conditions: 1, Average normal; 2, fast normal; 3, pregnancy, late; 4, malignancy; 5, tuberculosis; and 6, acute inflammation.

Profuse sweating without an opportunity to replace the water lost appears to raise viscosity by about 25 to 30 per cent. Measurement of viscosity is comparatively simple if one has a suitable instrument. The Hess instrument is one of the best and is accompanied by directions for use.

4. Sedimentation Speed of Red Corpuscles.—An increased tendency to sedimentation of red corpuscles in shed blood in certain pathologic conditions, particularly inflammation, has long been recognized as an interesting phenomenon explaining the well-known "buffy-coat" of coagulated blood. Within the last few years, following the work of Fahraeus, and others, with blood which has

been rendered non-coagulable by citrate or oxalate, the rate of sedimentation has been actively studied and applied clinically.

It has been found that the corpuscles settle more rapidly in the blood of women than of men, and very much more rapidly in pregnancy after the third or fourth month. Increased speed of sedimentation is also seen in tuberculosis, where it increases with the activity of the disease; in cancer, where it more or less closely parallels the degree of malignancy; and in localized acute inflammations, where the rate appears to increase with the leukocyte count.

A decided increase is practically limited to these conditions. Typical rates are shown in Figure 169.

The cause of the phenomenon is not clear. It is apparently connected with the ratio of albumin, globulin, and fibrinogen in the plasma, or with the concentration of cholesterol.

Since a variety of methods have been used the figures of different workers are not comparable. Some record the time required for the corpuscles to settle to an arbitrarily fixed point. Others note the height of the corpuscular layer after the blood has stood for a definite time, or make several readings at stated intervals and plot a curve.

The method given below is that of Cooper.

Determination of Sedimentation Rate (Cooper's Method).—1. Obtain blood from a vein as described for blood chemistry on page 333.

2. Place the oxalated blood in a graduated centrifuge tube exactly to the 5 c.c. mark.

3. Stand the tube upright in a rack.

4. Record the height of the red corpuscles at the end of five, ten, fifteen, thirty, forty-five, and sixty minutes, and then revolve the tube in a centrifuge at high speed for ten minutes. The percentage relation of corpuscles to the total column of blood are best recorded in the form of a graph (Fig. 169).

XV. SPECIAL BLOOD PATHOLOGY

Because of the special functions of the blood and because of its circulation in all parts of the body, every local or generalized disease will produce some change in it even though this may be inappreciable by our present methods. The great majority of blood

changes are, therefore, purely secondary and have no claim to be classed as blood diseases. Such of these changes as are significant in the diagnosis or prognosis of the underlying disease are discussed in previous sections of this chapter. There are, however, very few blood conditions (for example, malaria) which may be regarded as true blood diseases, and still others of which the etiology is so obscure and in which the blood changes are so conspicuous and so dominate the clinical picture that they are commonly spoken of as blood diseases. These will receive brief consideration in this section. In a general way they fall into four groups. In the first the characteristic feature is a change in the amount of hemoglobin and number of red corpuscles, either diminution (anemia) or increase (erythremia); in the second the leukocytes are chiefly affected (leukemia); in a third the clotting mechanism is disturbed (hemorrhagic diseases); and in a fourth there are animal parasites living in the blood (malaria and others).

A. Anemia

For the sake of simplicity anemia may be characterized as a deficiency of hemoglobin. The number of red corpuscles is diminished also, and decrease of blood volume and changes in the composition of the plasma are usual, but the hemoglobin loss is the most conspicuous change. *An estimation of hemoglobin is the most sensitive index of the existence and grade of anemia, and the most practicable;* but the normal variations dependent upon age and sex must be considered, as well as the inaccuracies of the instruments in common use. The appearance of the patient is often deceptive, since the color of the skin depends as much upon the capillary circulation as upon the color of the blood.

Factors which Determine the Blood-picture in Anemia.— Red corpuscle formation and red corpuscle destruction go on continuously within the body. In health there is such a balance between the two processes that the red corpuscles and the hemoglobin are maintained at a remarkably constant level. Any disturbance of this "hemogenic-hemolytic balance" results in an increase or a decrease of the hemoglobin percentage and the red cell count. When the balance is upset by deficient blood formation, on the one hand, or by excessive destruction or actual loss of blood upon the other, the result is anemia; and, in any given case, the red cells

and hemoglobin will rise or fall as the hemogenic-hemolytic ratio fluctuates. The red corpuscle count and the hemoglobin estimation therefore tell us no more than the grade of the anemia, that is, the resultant of the two opposing processes of hemogenesis and hemolysis. We can, in a certain crude way, get at the real conditions by a study of urobilin excretion, particularly in the feces or duodenal contents (p. 424), which serves as an index of blood destruction, and by a count of the percentage of reticulated red corpuscles (Vital Staining, p. 358), which furnish a guide to the activity of blood regeneration. Much the same information is given by Schneider's formula (p. 414) which combines the red cell count and the urobilin excretion.

In addition to simple diminution of hemoglobin and red corpuscles in anemia certain qualitative changes appear and may be very marked in the severer cases. In general these are an expression of the strain put upon the erythroblastic bone-marrow, which, when hard pushed, tends to react excessively and sometimes in an abnormal manner, and therefore puts out variable numbers of immature and imperfect red corpuscles (poikilocytes, polychromatophilic cells, nucleated red cells, and so forth). The presence of immature forms may be thought of partly as representing a lowering of the threshold whereby they overflow more easily into the circulating blood. In some anemias, owing probably to a toxic injury to the erythroblastic tissue, the type of blood regeneration takes on some of the characters of that seen in fetal life, and megaloblasts appear in great numbers in the bone-marrow and even pass over into the circulating blood. When an abundant supply of building material is available the bone-marrow may build an excess of hemoglobin into the individual red corpuscles, giving a high color index. This is possibly not the whole explanation of high color index, but is useful to keep in mind, since the condition is practically limited to the hemolytic anemias, in which the iron-containing portion of the liberated blood-pigment is retained in the body while the non-iron containing portion is eliminated as urobilin. When, upon the other hand, the iron has been lost to the body by hemorrhage, or when the bone-marrow is unable to utilize the materials at hand, the red corpuscles are deficient in hemoglobin and the color index is consequently low. Thus the lowest color index is found in posthemorrhagic anemia and in chlorosis.

On the basis of the above considerations the anemias may be divided into the following groups. The classification is based upon one proposed by Vogel. It is more or less arbitrary, but until more is known of their etiology will serve a useful purpose in making clear the nature and relations of the different forms. It will be noted that many clinical cases, for example, carcinoma with hemorrhage, fall into more than one class.

I. Anemia due to active destruction of red corpuscles within the circulation, either through strong hemolytic poisons or through excessive fragility of the red cells. These are known as "hemolytic anemias." The distinction from some cases in Group IV chiefly is one of *degree* of hemolysis, but is generally fairly sharp. *Urobilin of feces is very high and urobilin appears in the urine.*

1. With the blood-picture of pernicious anemia, including megaloblastic type of blood regeneration.
   (*a*) Progressive pernicious anemia.
   (*b*) Severe dibothriocephalus anemia.
   (*c*) Rare cases of anemia due to carcinoma, syphilis, or puerperal toxemia, with production of a strongly hemolytic poison.
   (*d*) Rare cases of hemolytic jaundice.
2. Without the blood-picture of pernicious anemia, the difference probably due partly to the time element, partly to the nature of the hemolytic agency.
   (*a*) Most cases of hemolytic jaundice (excessive fragility of red cells).
   (*b*) Malaria (parasites destroy red cells).
   (*c*) Hemolytic poisons: mushrooms, potassium chlorate, nitrobenzol.

II. Anemia due to loss of blood from body. *Urobilin of feces low.* Active regeneration of normal type.
   (*a*) Anemia of acute hemorrhage.
   (*b*) Anemia of chronic hemorrhage. Regeneration is active until, in extreme cases, the bone-marrow becomes exhausted.

III. Anemia due essentially to failure in hemogenesis. *No increase of urobilin in feces.* Blood regeneration slight or absent or of a defective type.
   (*a*) Chlorosis, hemogenesis of defective type possibly due to anomaly of internal secretion of female sex glands.
   (*b*) Aplastic anemia, hemogenesis slight or absent, due probably to toxic injury of bone-marrow.
   (*c*) Benzol poisoning.

The anemias grouped above are clear examples of the effects of excessive hemolysis, of hemorrhage, or of defective hemogenesis. There remain many others in which two or all of the three factors act together, each only to slight or moderate degree, though their combined effect may be very marked. This requires addition of a fourth group which overlaps the others to some extent, but which in practice generally gives fairly distinct clinical and hematologic pictures.

IV. Anemia due to moderately increased hemolysis, together with diminished bone-marrow function or destroyed bone-marrow. *Urobilin of feces not appreciably increased.* Evidences of compensatory regeneration present, but variable and nearly always of normoblastic type.

(*a*) The vast majority of the secondary anemias (acute and chronic infections, malignancy).

(*b*) The anemia of leukemia.

(*c*) Bone-marrow tumors, primary or secondary, the hemogenic tissue being crowded out. In extreme cases fetal blood-forming tissues (liver, and possibly other organs) may resume function with formation of megaloblasts.

Clinically it is customary to speak of *primary* and of *secondary anemia*. Secondary anemia is symptomatic of some other pathologic condition. Primary anemia, which includes progressive pernicious anemia, chlorosis, aplastic anemia, and hemolytic jaundice, appears to develop and progress independently of any other disease. The classification is not satisfactory, but has long been current and serves a useful purpose. It is not always possible to distinguish between the two groups, although each type has a more or less distinctive blood-picture. Not infrequently autopsy shows a supposed primary anemia to have been really secondary, usually due to malignancy.

1. Secondary Anemia.—The more important conditions which produce secondary or symptomatic anemia are listed below. In many clinical cases two or more of these operate together:

(*a*) *Poor nutrition*, which usually accompanies unsanitary conditions, such as poor and insufficient food.

(*b*) *Acute infectious diseases*, especially rheumatism and typhoid fever. The anemia is often more conspicuous during convalescence.

(*c*) *Chronic infectious disease;* tuberculosis, syphilis, leprosy, chronic suppuration.

(*d*) *Chronic exhausting diseases*, as chronic nephritis, cirrhosis of the liver, and gastro-intestinal disease, especially when associated with atrophy of gastric and duodenal glands.

(*e*) *Chronic poisoning*, as from lead, arsenic, or phosphorus.

(*f*) *Hemorrhage*, either repeated small hemorrhages (chronic hemorrhage), as from gastric or uterine cancer, gastric ulcer, hemorrhoids, uterine fibroids; or acute hemorrhage such as may occur in typhoid fever, tuberculosis, abortion, or traumatism.

(*g*) *Malignant tumors*, particularly in the gastro-intestinal tract and the uterus.

(*h*) *Animal Parasites.*—Some cause no appreciable change in the blood; others, like the malarial parasite, the hookworm, and the "fish tapeworm," *Diphyllobothrium latum*, may give rise to very severe anemia, which in the case of *D. latum* closely simulates progressive pernicious anemia. *Examination of feces for the ova of parasites should never be omitted in cases of obscure anemia.*

Most but not all of the secondary anemias fall into the fourth group of the classification previously given.

The blood changes vary somewhat with the cause, but in general the picture is much the same for cases of secondary anemia of equal severity. Diminution of hemoglobin is the constant and most characteristic feature. In mild cases it is slight and is the only blood change to be noted; in these cases the normal hemoglobin variations dependent upon age and sex must be taken into account as well as the inaccuracies of the usual clinical hemoglobinometers. A reading which is normal for a girl of seven years would mean anemia for a man. In moderate cases of anemia hemoglobin ranges about 60 to 75 per cent.; in severe cases, about 40 to 50 per cent.; while in extreme cases it may fall to 15 per cent. or even lower. Red corpuscles are diminished in all but very mild cases, while in the severest cases the count is sometimes as low as 1,000,000 for each cubic millimeter. The color index is generally reduced to about 0.7 or 0.8, but may rarely fall to 0.5. Secondary anemia with exceptionally low color index is most frequently due to hemorrhage and is spoken of as "secondary anemia of the chlorotic type."

Although the number of leukocytes bears no direct relation to the anemia, polymorphonuclear leukocytosis is common, being due to the same cause as is the anemia.

Stained films show no appreciable changes in very mild cases.

In moderate to severe cases variations in size and shape of the red cells occur, while scattered polychromatophilic cells are found, and even occasional normoblasts. Very severe cases show the same changes to greater extent, with addition of basophilic granular degeneration and the presence of normoblasts in small or considerable numbers. However, the student must be cautioned that, even when "very numerous," these abnormal cells can usually be found only after a search; they are not found in every field, as many seem to expect. The number of normoblasts bears no direct relation to the severity of the anemia, but rather serves as a rough index of the regenerative activity of the bone-marrow. At times, particularly in acute posthemorrhagic anemia, great numbers of normoblasts may appear rather suddenly—a so-called *blood crisis*—and this is often followed by rapid increase in the red count. The finding of more than five nucleated red cells for each hundred leukocytes seen during the differential count is rather arbitrarily taken to indicate a blood crisis. Megaloblasts in small numbers have been encountered in certain severe cases of secondary anemia, but in interpreting reports one should know the criteria adopted in the particular case for identification of the megaloblast. They are especially numerous and may even exceed the normoblasts in severe anemia of Diphyllobothrium infestation, which gives a blood-picture identical with that of pernicious anemia, and in the anemia of malignant disease, presumably with metastases in the bone-marrow. Crowding out of the hemogenic tissue of the bone-marrow apparently causes the liver to resume to some extent its fetal function of manufacturing red corpuscles, with the result that red cells of the fetal type, megaloblasts, reach the circulation.

In the anemia of lead-poisoning a striking feature and one of much diagnostic significance is the exceptionally large number of red cells which show blue granulation with the polychrome methylene-blue-eosin stains (Fig. 170). This granulation is probably not identical with that seen in other anemias, being apparently due to the presence of lead in the corpuscle. These granulated cells may be found in chronic and acute lead-poisoning even when little or no anemia is apparent, while in cases with marked anemia they may be so numerous that several appear in every microscopic field. Polychromatophilic cells are generally more numerous than in the usual anemias.

Acute posthemorrhagic anemia deserves more extended mention. The greatest amount of blood which can be lost at one time with recovery depends upon the age and health of the individual, therapeutic measures, and other factors, but is generally taken to be about one-half the total blood-volume. Immediately after a large hemorrhage the red cell count will be the same as before. Within a few hours the volume of blood is, to a great measure, restored by means of fluids from the tissues, and the process of dilution continues more slowly for a day or two. At the same time the red cell count and hemoglobin percentage fall to a figure roughly corresponding to the amount of blood lost. Active

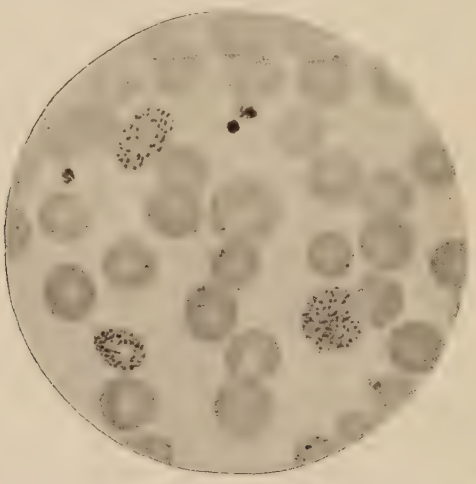


FIG. 170.—Blood from a case of secondary anemia due to lead-poisoning. In this small area note three red corpuscles showing basophilic granular degeneration (photograph, × 1000).

regeneration of corpuscles begins within a short time, probably within twenty-four hours. The new corpuscles tend to be deficient in hemoglobin, hence the color-index is low, sometimes strikingly so. Some of the newly-formed cells show polychromatophilia, and a few normoblasts commonly reach the circulation. Occasionally blood crises occur, with subsequent improvement in the blood-picture. There is moderate neutrophilic leukocytosis. Blood-platelets are strikingly increased, even up to 1,000,000 for each cubic millimeter.

The time required for restoration of the number of corpuscles is about a month in the case of a large hemorrhage in a previously healthy person. After a loss of 500 to 600 c.c.—an amount fre-

quently given by donors in transfusion cases—the number of corpuscles is usually made up in one or two weeks, although the hemoglobin percentage lags behind. Giffin and Haines have shown that professional donors, who are usually between the ages of twenty and forty, may give blood every four to six weeks for two years or more with no apparent ill effects, and even with improvement in general health and increase in weight. In some, however, repeated bleedings are followed by mild anemia to which women show a greater tendency than do men; and in a very few, probably because of preëxisting weakness of the blood-forming tissues, marked chronic anemia may develop.

2. Progressive Pernicious Anemia (Addison's Anemia).—Whether this is an entity, or whether it represents a group of conditions with similar pathogenesis but different etiology, is at present not fully settled. The essential feature is excessively active destruction of red corpuscles by an unknown poison, together with active, although abnormal, regeneration of red corpuscles by the bone-marrow. The course of the disease and the blood-picture vary with the varying rate of blood destruction, with the functional sufficiency of the hemogenic bone-marrow, and with the bone-marrow threshold for immature cells. As a terminal event the bone-marrow may become exhausted, giving a blood-picture similar to that of aplastic anemia. Characteristic of the clinical course is a striking tendency to remissions, which may occur spontaneously or may be induced by one or another therapeutic measure. At such times hemolysis is greatly reduced, and the blood-picture approaches the normal. There may be one or several of these remissions, lasting from a few weeks to many months or even years; but eventually the case relapses and finally terminates fatally. Stockton has reported a case with remission of twelve years. While in some cases diagnosis can be made with considerable certainty from a study of repeated blood examinations, yet, as a rule, a careful analysis of all clinical and laboratory data is required, and even this may sometimes be indecisive. Of the laboratory findings the most significant for diagnosis are the blood-picture, the great increase of urobilin in urine and feces, and the absence of free hydrochloric acid from the gastric juice. During remissions diagnosis may be impossible.

Pernicious anemia is practically never seen at the onset of the

disease, so that red cells are generally very low when the first examination is made—usually 1,500,000, or less, for each cubic millimeter in dispensary work, more frequently about 2,500,000 in private practice. There seems to be a tendency for the red cells to fall rapidly to a certain level, which varies with the case, usually between 1,000,000 and 1,500,000 for each cubic millimeter, and to remain fairly stationary near this level. However, a few cases have been reported which terminated fatally with the red corpuscles above 2,500,000 for each cubic millimeter, while, upon the other hand, counts as low as 150,000 have been recorded. In remissions, especially those following blood transfusion, the red count often rises with surprising rapidity, for example, from 1,000,000 to 3,000,000 within a few days.

The average hemoglobin value in active cases is about 20 to 25 per cent. In more than two-thirds of the cases the hemoglobin loss is less than the loss of red cells: the color-index is therefore high, usually 1.1. to 1.5, sometimes as high as 2. A color index of 0.8 or below speaks against a diagnosis of progressive pernicious anemia, although it may occur in mild cases, or in the early stage of a remission when the blood is rapidly regenerating, as following transfusion. To be of any value, however, color-index determinations must be based upon an accurately standardized hemoglobinometer (p. 252). The volume index is high, and is even more significant than is the color index.

In stained films red corpuscles show marked variations in size and shape. There is decided tendency to large oval forms, and despite the presence of microcytes the average size of the corpuscles is generally strikingly increased. In some cases even the majority exceed 11 μ in diameter, a circumstance of great diagnostic significance. A few of these large cells show endoglobular degeneration. Another striking feature of the red cells is absence of achromia. An occasional corpuscle may show marked pallor, but the great majority evidently contain their full amount of hemoglobin, and many are more deeply and solidly tinted than normal. Polychromatophilic red cells and cells showing basophilic granular degeneration, especially the former, are numerous in some cases, scarce in others. Nucleated red corpuscles are probably present in every case, though not always continuously. Contrary to the impression usually gained by students who in their class work have studied

only slides from selected cases, nucleated red corpuscles are not often numerous, a search of fifteen minutes to an hour or more being generally required to find them. As a rule, the presence of a large number is a point against the diagnosis of pernicious anemia,

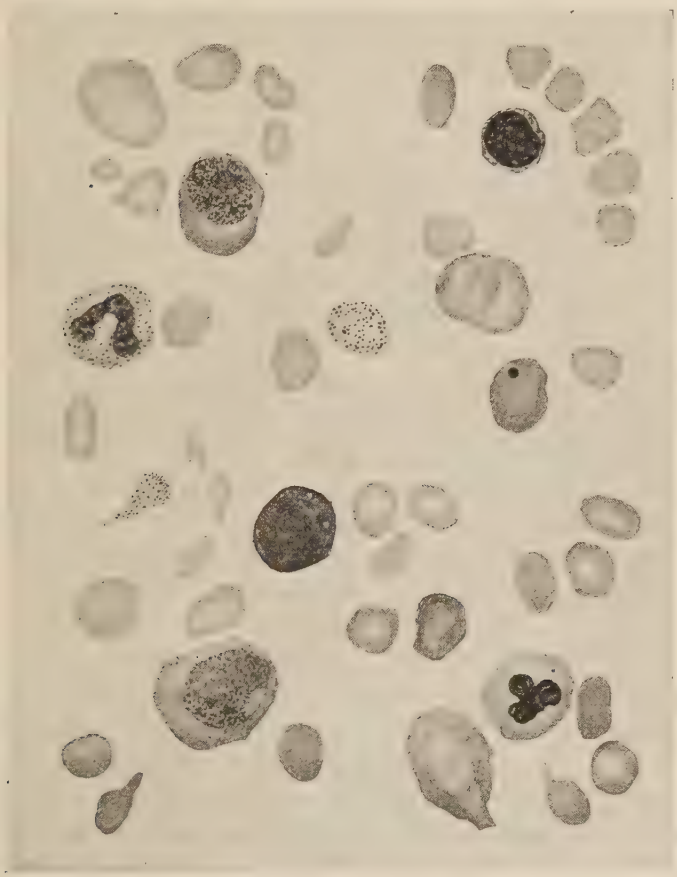


FIG. 171.—Blood-cells in pernicious anemia. Note variations in size and shape of the red corpuscles; three megaloblasts, one with irregular, deeply stained nucleus; red corpuscles showing grades of polychromatophilia, basophilic granular degeneration, and one nuclear particle; one lymphocyte, and one polymorphonuclear neutrophil. All drawn from actual cells on two slides. Wright's stain; × 800 (0.8 mm. = 1 micron).

except during a blood-crisis, which is an infrequent incident. They are of both normoblastic and megaloblastic type, and, in many cases, perhaps the majority, megaloblasts exceed normoblasts in number. This ratio is most unusual in the occasional case of secondary anemia which may show megaloblasts. The diagnostic

weight given megaloblasts by different workers varies, but in general less importance is ascribed to them than formerly. It depends in a large measure, upon the criteria adopted for identifying these cells (p. 281). The young megaloblasts, larger than polynuclear leukocytes, nearly always polychromatophilic, and with large delicately reticular nuclei, are indeed very rarely seen in other conditions; but in probably half of the cases of pernicious anemia they are found only after tedious search or not at all. Sometimes they are unevenly distributed, few or none being found on one slide, while on another, made at the same time, they may be readily found. In many cases of pernicious anemia only the older megaloblasts, with small, condensed nuclei, can be found in any length of time which is practicable in clinical work; and these have less significance, although still very suggestive (Fig. 171).

The leukocyte count may be normal or moderately high, but is commonly diminished to about 3000 or 4000 for each cubic millimeter, and is sometimes much lower. The decrease affects chiefly the neutrophils so that lymphocytes are relatively increased. In some cases a decided absolute increase of lymphocytes occurs. Neutrophilic leukocytosis, when present, is due to some complication. Occasional neutrophilic myelocytes can usually be found. L. H. Briggs reports a low Arneth index (p. 293) in 10 of 12 cases studied.

Vital staining (p. 358) will generally demonstrate a marked increase in the number of reticulated red corpuscles which usually range between 1 and 5 per cent. in this disease, but may at times reach 20 per cent. of all the red corpuscles. This percentage is a useful index of the activity of blood regeneration, and it should be considered in connection with the urobilin excretion, the index of hemolysis, since it is upon the balance between hemogenesis and hemolysis that the welfare of the patient depends. Resistance of the red corpuscles to hypotonic salt solutions (p. 363) is not much changed, but is usually slightly increased, thus showing that the excessive hemolysis is not due to increased fragility of the red cells as it is in the case of hemolytic jaundice.

As a rule blood-platelets are diminished, even below 50,000 in some cases. The decrease is apparent in the stained film, where it may be difficult to find any. Coagulation time is delayed.

To sum up the blood findings in typical cases of pernicious

anemia: the red cells are greatly diminished, the color index and volume index high, leukocytes normal or slightly diminished, platelets reduced. Many macrocytes, and a few megaloblasts are present in stained films.

Scarcely less important than the blood-picture, and always to be studied in connection with it, is the urobilin content of urine, feces, and duodenal contents, which serves to distinguish the hemolytic group of anemias from those in which increased hemolysis is not an important factor. In pernicious anemia the urobilin of the feces usually reaches 20,000 to 30,000 or more dilution values by the Wilbur and Addis method. During remissions urobilin falls, and may go to normal.

Another feature of very great importance is the early disappearance of free hydrochloric acid from the stomach contents, sometimes long before any anemia is recognizable. When once established, achlorhydria persists, even during remissions in which the blood appears to be practically normal.

There is recognized a **hemolytic anemia of pregnancy** which has the blood-picture of pernicious anemia, although there is less tendency to macrocytosis and high color-index. Gastric hydrochloric acid is low, but not entirely absent. The disease is of rare occurrence. It is less fatal than is pernicious anemia, and when once overcome does not tend to recur.

3. Sickle-cell Anemia.—In 1910 J. B. Herrick described a peculiar form of anemia in which a large proportion of the red corpuscles were crescentic or fusiform in shape. This anemia is now recognized as a clinical entity, and many cases are on record, all in negroes. It is a hereditary and familial disease, present and recognizable very early in infancy. There is probably a congenital defect of the red corpuscles which renders them susceptible to certain hemolytic poisons, and to phagocytosis, although resistance to hypotonic salt solutions appears to be normal or in some cases slightly increased. The disease appears in two forms, latent and active. While transitions from one to the other may occur, these are not merely stages in the development of the disease.

In the latent form, which is much the more common, there is no definite anemia. The usual routine blood examination shows no more than anisocytosis with many microcytes, and, on careful search, a few endothelial leukocytes containing phagocytized red

cells. The diagnosis is established only by the study of wet preparations sealed with vaseline, and kept in a warm place. After eighteen or twenty-four hours these show great numbers of the remarkable crescentic fusiform and stellate red corpuscles which are characteristic of the disease, and also active phagocytosis of red cells by endothelial leukocytes.

In the active form, which is most active in children, anemia is marked. Red corpuscles usually number 1,500,000 to 3,000,000 in each cubic millimeter. Many sickle-shaped and elongated corpuscles are present in stained films (Fig. 172), and phagocytosis of red cells by endothelial leukocytes, and even neutrophils, is always present. These characteristics become much more marked in sealed wet preparations which stand for some hours. There are many microcytes and numerous normoblasts. Vital staining shows a great increase of reticulated red corpuscles, which reach 15 to 40 per cent. of the total number. Moderate to marked leukocytosis is the rule with nearly normal differential count, and ordinarily a shift of Arneth's formula to the right; a few macrocytes may be present. There is much urobilin in urine and feces, showing excessive blood destruction. The acidity of gastric contents is low, but hydrochloric acid is not completely absent.

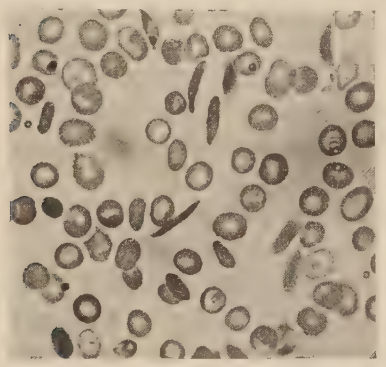
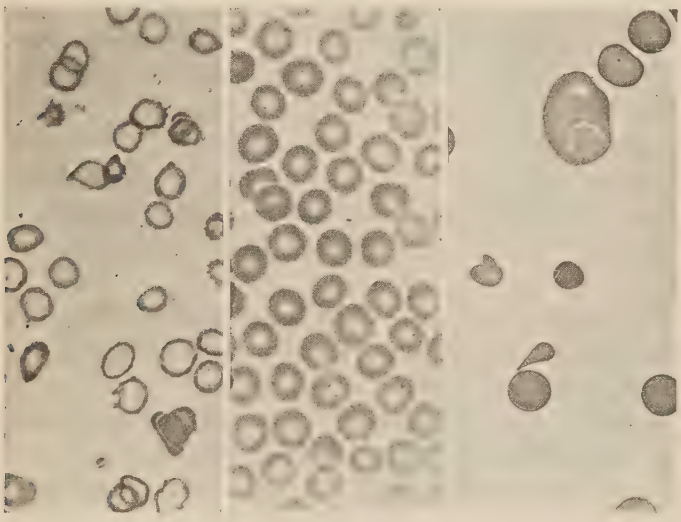


FIG. 172.—Blood in sickle-cell anemia, active form, stained film. The diagnosis is best made from unstained wet preparations, in which, after a few hours, the number of crescentic and stellate forms is greatly increased. (Photograph about × 500.) (Courtesy of V. P. Sydenstricker.)

4. Chlorosis.—This is essentially an anemia of defective blood formation, which is confined almost exclusively to girls within the decade following the age of puberty. The name was suggested by the peculiar greenish pallor which typical cases exhibit. While the clinical symptoms furnish the most important data for diagnosis, a consideration of the blood findings is essential. The most conspicuous feature is a *marked* decrease of hemoglobin accompanied by a *slight* decrease in number of red corpuscles, this giving a strikingly low color-index with marked achromia of the red corpuscles in stained films.

The following figures represent about the average for well-marked cases: hemoglobin, 35 to 40 per cent.; red corpuscles, 3,500,000 to 4,000,000 in each cubic millimeter; color index, 0.4 to 0.5. Mild cases may have only a marked loss of hemoglobin, the number of red cells remaining normal. In the severer cases, upon the other hand, red cells may fall below 2,000,000, and the color index to 0.3. It is extremely rare for any secondary anemia—even posthemorrhagic—to give a color-index below 0.5.

In contrast to pernicious anemia (and in some degree also to secondary anemia), the cells are of nearly uniform size; their



A B C

FIG. 173.—Red blood-corpuscles in chlorosis (A) and pernicious anemia (C) contrasted with those of normal blood (B). In a well-marked case of chlorosis the red corpuscles are pale and ring-like; in pernicious anemia they are rich in hemoglobin, and show marked variations in size and shape. The megalocyte in the upper part of the figure is especially characteristic of pernicious anemia. Wright's stain; photographs (× 750).

average diameter is somewhat less than normal; and the central pale area is unusually large and pale (achromia), giving the cells a striking ring-like appearance (Figs. 127 and 173). Only in severe cases do polychromatophilia, and marked changes in shape occur. Erythroblasts are rarely found. The number of platelets is generally increased, and the blood coagulates rapidly.

As in pernicious anemia the leukocytes are normal or decreased in number. The differential count generally gives normal figures or a moderate increase of the lymphocytic percentage.

5. Acute Aplastic Anemia.—There is a rare and rapidly fatal anemia of obscure etiology which is apparently the result of more or less complete failure of blood formation, the red bone-marrow being found at autopsy to have almost wholly disappeared, even from the flat bones and the bodies of the vertebræ. To this the name "aplastic anemia" is applicable. The failure appears to involve red blood-corpuscles first, then leukocytes, and finally platelets.

The disease is most frequent between the ages of sixteen and thirty; its duration is only about two or three weeks. The red corpuscles sink to 1,000,000 for each cubic millimeter or less, and hemoglobin likewise falls rapidly. The leukocytes generally are low. Platelets are diminished, and when they are very low symptoms like those of purpura hæmorrhagica appear.

In contrast to other conditions which give so low a red cell count, stained films show little that is abnormal unless some foci of active bone-marrow remain, in which case a few normoblasts and other cells indicating attempted blood regeneration may be found.

Reticulated red cells are rare; resistance of the red cells to salt solutions of various strengths is about normal. Bleeding time and coagulation time are somewhat prolonged.

Aplasia of the bone-marrow, with a similar blood-picture, may occur secondarily in leukemia and other conditions.

6. Hemolytic Jaundice (Congenital Familial Icterus).—This is a rare, chronic, generally hereditary disease, characterized by periods of excessive hemolysis, due in part at least to abnormal fragility of the red corpuscles. There is marked splenomegaly.

The most striking feature from the laboratory point of view is the lowered resistance of the red cells to hypotonic salt solution, initial and complete hemolysis, in a typical, well-marked case, occurring in salt solution of 0.5 and 0.4 per cent. respectively (p. 363). Definitely lowered resistance may, in fact, be regarded as almost certain evidence of the existence of hemolytic jaundice. Only in exceptional mild cases is the resistance normal, and probably not at all times in these. The anemia is usually very marked, with the red cells between 1,500,000 and 3,000,000 in each cubic millimeter, and the color-index about 0.8 to 0.9. Sometimes the anemia takes on the character of progressive pernicious anemia.

Urobilin of urine, feces, and duodenal contents is high; and vital staining shows a distinctly higher percentage of reticulated red cells than is usual in pernicious anemia.

B. Polycythemia

Secondary increase of red corpuscles and hemoglobin due to chronic heart disease, concentration of blood in severe diarrheas, and other causes, is known as erythrocytosis, and has been discussed (p. 241). In addition, a rare "idiopathic polycythemia," possibly somewhat analogous to leukemia, is recognized, and is known as erythremia or polycythemia rubra. There is marked hyperplasia of the red bone-marrow, thought by some to be of the nature of a malignant tumor arising from the erythroblast, and the spleen is generally much enlarged. Patients exhibit a peculiar and striking cyanotic cast of countenance. Minot and Buckman have observed an apparent transition from erythremia to myelogenous leukemia in three cases, and believe that the two diseases are intimately related.

The red corpuscles of the circulating blood number 7,000,000 to 12,000,000. The highest counts recorded are 15,500,000 and 15,900,000. Hemoglobin ranges from 110 to 150 per cent.; and the color index is moderately low. Macrocytes, microcytes, polychromatophilic red cells, and normoblasts may be met, but are not prominent. Moderate polymorphonuclear leukocytosis is the rule. The blood coagulates rapidly. As would be expected its viscosity is very high. The total volume of the blood is two to three times the normal. Resistance of red corpuscles to hypertonic salt solutions has been found increased when tested. R. J. Pickard has noted an increased resistance of the red cells to antihuman hemolytic amboceptor.

C. Leukemia

Two types of the disease are commonly distinguished. Atypical cases are not rare, especially in children. The disease is characterized by hyperplasia and overactivity of the leukoblastic bone-marrow (myelogenous leukemia) or of the lymphoid tissue (lymphatic leukemia), together with overflow of many immature leukocytes, and excessive numbers of the adult normal types into the circulating blood. By some it is regarded as a neoplasm with metastases in the blood-stream. An acute and a chronic form of each of the

two types occur, although chronic cases are by far the more common. In general, the more acute the process the larger the proportion of very immature cells which reach the circulation.

1. Chronic Myelogenous Leukemia.—Owing to its insidious onset, the disease is rearely recognized until well established. By this time, except in rare instances, the diagnosis is easily made from the blood alone, usually at the first glance at the stained film. Not infrequently the existence of the disease is first revealed by a blood examination made with some other diagnosis in view. A significant clinical feature, which may be the first sign to arouse suspicion, is great enlargement of the spleen; because of this the name splenomyelogenous leukemia is sometimes used. The duration of the disease is variable; it is usually fatal within two to five years, but cases which were under observation for a much longer period are on record.

The most striking feature of the blood-picture is an enormous increase in number of leukocytes, which is usually evident at the first examination. The leukocyte count in ordinary cases lies between 100,000 and 400,000 in each cubic millimeter, while counts over 1,000,000 have been met. In exceptional cases, upon the other hand, the counts may not rise above 50,000. The height of the count is not necessarily an index of the severity of the disease. There is little tendency to progressive increase as the disease advances, and there may even be a decided fall in the count in the terminal stages. During spontaneous remissions, during intercurrent infections, and during treatment with *x*-ray or benzol, it may fall to normal.

While these enormous leukocyte counts are approached in no other disease except lymphatic leukemia, and a rare case of extremely high-grade neutrophilic leukocytosis, the diagnosis, particularly during remissions, depends more upon qualitative than quantitative leukocytic changes (Plate VIII, Fig. 2). Although all varieties are increased, the characteristic and conspicuous cell is the myelocyte, and diagnosis cannot be made in its absence. This cell never appears in normal blood, rarely in leukocytosis or lymphatic leukemia, and only occasionally in pernicious anemia. In chronic myelogenous leukemia it usually constitutes more than 20 per cent. of all the leukocytes. DaCosta's lowest case had 7 per cent. The neutrophilic form is generally much more abundant than

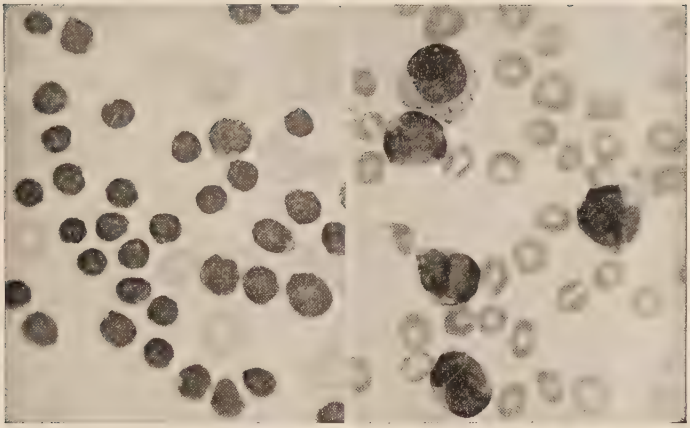


FIG. 1.—Blood in lymphatic leukemia; × 700. On the left, chronic form of the disease; on the right, acute form (courtesy of Dr. W. P. Harlow).

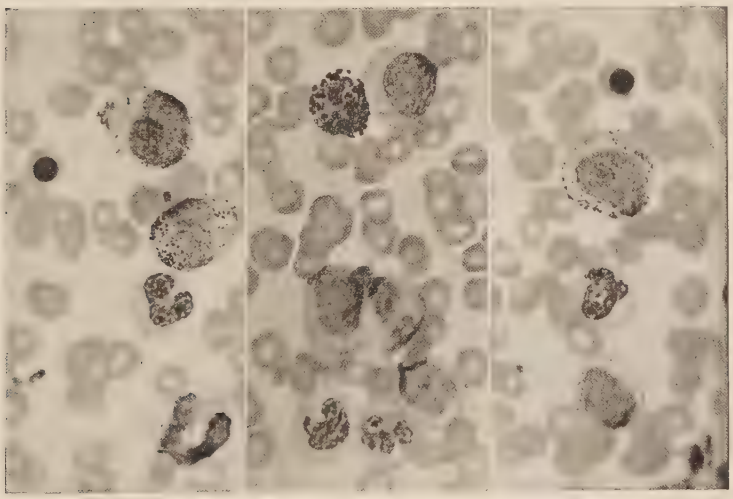


FIG. 2.—Blood in chronic myelogenous leukemia. Wright's stain; × 700.

the eosinophilic, which, however, is the more characteristic, as it occurs practically nowhere else. Both show considerable variation in size. Myeloblasts may be present in small or moderate numbers at any time, and in the terminal stage may be abundant. A marked increase in their number is of grave significance, indicating a tendency to a more acute course. Very constant, though not of much diagnostic significance, is a marked absolute, and often a relative increase of eosinophilic and basophilic leukocytes. Polymorphonuclear neutrophils are absolutely increased, although relatively decreased. Many atypical and degenerated leukocytes, which may be listed as "unidentified," may be found.

When the patient is first seen the red cell count may be normal with only slight deficiency of hemoglobin. Sooner or later, however, a definite anemia develops, the red cells generally falling below 3,500,000. Accurate estimation of hemoglobin is difficult in some cases, owing to the cloudiness produced by the great number of leukocytes. The color index is moderately low. In stained films the red cells show the usual changes seen in severe secondary anemia, excepting that nucleated red corpuscles are commonly very numerous. In fact, no other disease gives so many or offers so good an opportunity for their study. They are chiefly of the normoblastic type, and often all stages in their aging can be followed, from large polychromatophilic, immature forms which resemble megaloblasts to small orthochromatic forms with pyknotic nuclei. Mitotic figures are common. True megaloblasts are present in some cases, but it is often difficult to decide whether a given cell is a megaloblast or an exceptionally young normoblast. Blood-platelets are variable, but generally greatly increased.

2. Acute Myelogenous Leukemia.—This is a rare form of leukemia which rapidly progresses to a fatal termination, usually within three to eight weeks. Prominent clinical symptoms are irregular fever, and a marked tendency to hemorrhages from gums and mucous membranes and into the skin. In some cases there is a throat condition closely resembling Vincent's angina, and containing the typical spirochete.

The leukocyte count is not so high as in chronic myelogenous leukemia, seldom exceeding 100,000 in each cubic millimeter; and it may be below 10,000. The predominating leukocyte is the most immature member of the bone-marrow series, the myeloblast.

Since, morphologically, this cell closely resembles the large lymphocyte of acute lymphatic leukemia, it may be impossible to distinguish between the two types of leukemia by the blood examination. In general, the peroxydase reaction (p. 299) will serve to identify the megaloblast; but in some cases the cells are so very immature that the test fails. In most cases it is best to be content with a diagnosis of "acute leukemia," leaving the question of the precise nature open until cells of a more mature type are found. When the case is of myelogenous origin, a sufficient number of myelocytes can usually be found to put one upon the right track. Eosinophilic myelocytes would, of course, be especially significant.

The accompanying anemia, which develops rapidly, is generally severe, the red cells sometimes falling to 1,000,000; and in some cases, when the leukocyte count is low, there may be difficulty in excluding pernicious anemia.

3. Chronic Lymphatic Leukemia.—The course of the disease extends over years, sometimes as many as ten. An outstanding feature in most cases is a generalized enlargement of the lymph-nodes.

The leukocyte count is high, but less so than in chronic myelogenous leukemia. Counts of 100,000 are about the average, but in exceptional cases may go as high as 1,000,000. Upon the other hand, the count may be much lower, even as low as 15,000. Very rarely the leukocytes do not exceed the normal, and the name "aleukemic leukemia" may be applied. In such cases the diagnosis must be based upon the clinical findings, and the differential leukocyte count. Lymphocytes constitute 90 to 98 per cent. of the total number (Plate VIII, Fig. 1). As a rule, most of them resemble the small mature lymphocyte seen in normal blood, but large immature forms, like those in the blood of children, may be numerous in some cases, while atypical forms with deeply indented nuclei are common. Very frequently the lymphocytes appear to be abnormally fragile, since many, even the majority, may be ruptured in thin films (Fig. 147). During remissions, and sometimes near the end, the leukocyte count may fall below normal, but the percentage of lymphocytes remains high. Occasionally a stray myelocyte is encountered.

The red cells and hemoglobin may stand at a high normal level for a long time, but sooner or later anemia develops, and, as a

DIFFERENTIAL DIAGNOSIS OF BLOOD DISEASES

	Hemoglobin.	Red Corpuscle Count.	Color-index.	Leukocyte Count.	Stained Films. Red Corpuscles.	Stained Films. Leukocytes.
Secondary anemia.	Diminished according to degree of anemia.	Normal in mild cases; diminished in all others.	Normal or slightly diminished.	Not necessarily affected; leukocytosis common.	Variations in size and shape in moderate cases; variations in staining reactions and normoblasts in severe cases.	Normal proportions or increase of polynuclears.
Pernicious anemia.	Diminished.	Greatly diminished.	High.	Normal or diminished.	Marked variations in size, shape, and staining reactions. Average size increased. Tendency to large oval forms. Erythroblasts always present; megaloblasts exceed normoblasts.	Lymphocytes relatively, sometimes absolutely, increased.
Chlorosis.	Greatly diminished.	Slightly diminished.	Low.	Normal or diminished.	Nearly uniform size and shape; average size decreased; pale centers. Erythroblasts very rare.	Lymphocytes apt to be relatively increased.
Myelogenous leukemia.	Decidedly diminished.	Decidedly diminished.	Usually slightly diminished.	Extremely high.	Similar to secondary anemia, except normoblasts generally very numerous.	Large numbers of myelocytes (average, 20 per cent.). Absolute increase of eosinophils and basophils. Relative decrease of polynuclears and lymphocytes.
Lymphatic leukemia.	Markedly diminished.	Markedly diminished.	Usually slightly diminished.	Very high.	Similar to secondary anemia. Erythroblasts not numerous.	Lymphocytes exceed 90 per cent. Other varieties relatively decreased.

rule, is very marked. The color index is moderately low. The red cells show the usual changes of a severe secondary anemia. Erythroblasts are seldom abundant. Platelets are decreased in most cases.

4. Acute Lymphatic Leukemia.—This is rare, but is more frequently seen than is acute myelogenous leukemia, which it closely resembles in its symptomatology and clinical course. Even a study of the blood will not serve to distinguish the two in all cases, owing, as has been previously explained, to the close morphologic similarity between the lymphoblast and the myeloblast. In contrast to the chronic leukemias, which are diseases of adult life, acute leukemia is most frequent in childhood and youth.

As in all forms of leukemia the number of leukocytes varies in different cases, and fluctuates in the same case. The count may exceed 100,000 in each cubic millimeter in some cases; while in others it never goes above 15,000. At times it may fall below 5000 (aleukemic leukemia). Large, immature lymphocytes (lymphoblasts) predominate in the great majority of cases (Plate VIII, Fig. 1), although there is usually a sufficient percentage of ordinary lymphocytes to prevent confusion with acute myelogenous leukemia. Many of the lymphocytes, especially the large ones, are atypical, the form with lobulated nucleus (Rieder's form) being especially frequent.

Red cells fall rapidly, even to 1,000,000 in each cubic millimeter; and there is great loss of hemoglobin with usually a low color index. Normoblasts may be found.

D. Hemorrhagic Diseases

A marked tendency to hemorrhage from the mucous membranes and into the tissues and skin, with the formation of ecchymoses, is a prominent feature of a number of conditions. In some the hemorrhagic tendency is manifestly a symptom of a recognized disease. In another group, including purpura hæmorrhagica, hemophilia, and melena neonatorum, the bleeding tendency appears to be the primary feature of the disease. The immediate cause in both groups appears to be a disturbance of the clotting mechanism, whereby the coagulation time is prolonged or the clot is imperfectly formed. The causes underlying the defective clotting are not fully understood, but differ in the different diseases. There may be:

(*a*) deficiency in number of platelets, leading to the formation of a non-retractile clot, as in purpura hæmorrhagica, aplastic anemia, and lymphatic leukemia; (*b*) alteration of their function, leading to deficiency of available prothrombin, as in hemophilia, melena neonatorum, and aplastic anemia; (*c*) deficiency in amount of fibrinogen, as in cirrhosis, and some acute destructive diseases of the liver with hemorrhagic symptoms; (*d*) lack of available calcium, as in obstructive jaundice, in which the blood calcium appears to be bound by the bile-pigment, or (*e*) excess of antithrombin, which may be a factor in hemorrhagic septicemia and other conditions.

A hemorrhagic tendency may also result from weakness of the small vessels and from emboli.

Only the primary hemorrhagic diseases will be taken up in detail.

1. Purpura Hæmorrhagica.—The most striking and significant feature is an enormous decrease in number of blood-platelets. They range between 40,000 and 75,000 in each cubic millimeter in mild cases, and are reduced to 15,000 or even 10,000 in severe cases. The cause of the platelet loss is obscure and probably varies in different cases. Coagulation time is usually normal or nearly so, but the clot is soft and lacks retractility (p. 229). The bleeding time is very much prolonged, generally an hour, sometimes longer, and furnishes a direct index of the tendency to bleed. In some cases at least there may be abnormalities in other coagulation factors, and there is practically always toxic injury of the capillary endothelium which makes these vessels very fragile.

The severe anemia which develops is due to the loss of blood and differs from ordinary posthemorrhagic anemia chiefly in the number of platelets, which is increased in the latter condition. In general there is slight or moderate neutrophilic leukocytosis.

2. Hemophilia (*Bleeder's Disease*).—This is an interesting sex-linked hereditary tendency to hemorrhage confined to males, but transmitted by females who do not themselves show any tendency to abnormal bleeding. There are, however, many cases in which hereditary transmission cannot be established. The cause of the disease is apparently a congenital abnormality of the blood-platelets which are normal in number, but physiologically defective.[1]

[1] Minot, G. R., and Lee, R. I.: The Blood-platelets in Hemophilia, Arch. Int. Med., vol. 18, p. 474, October, 1916.

Howell and Cekada[1] recently showed that the platelets are more resistant than normal, and, contrary to the opinion formerly held, there is no change in the quantity or quality of the prothrombin. Nevertheless the so-called "prothrombin time" is five to twenty-five times the normal. This may be due to the slowness with which thromboplastin is liberated from the platelets. The coagulation time, when blood is taken from a vein, is about five to fifteen times the normal, that is, from one to five hours or longer. When blood is secured from a skin puncture, the coagulation-accelerating substances of the tissue juice may reduce the coagulation time nearly or quite to normal. When once formed, the clot is firm and has normal retractile power. The bleeding time by Duke's method (p. 232) is usually normal, which at first sight seems inconsistent with the well-known tendency of hemophiliacs to bleed from wounds. The difference apparently lies in the opportunity for admixture with tissue juice, which is relatively less in the case of large wounds or wounds of the mucous membranes.

3. Melena Neonatorum.—The condition is not well understood. In some cases, at least, the platelets have not been reduced and the bleeding time has been only slightly prolonged, but prothrombin time and coagulation time have been delayed.

E. Diseases Due to Blood Parasites

These have already been discussed (pp. 304–320).

[1] Howell, W. H., and Cekada, E. B.: The Cause of the Delayed Clotting of Hemophilic Blood, Amer. Jour. Physiol., vol. 78, p. 500, November, 1926.

CHAPTER IV

GASTRIC AND DUODENAL CONTENTS

I. EXAMINATION OF THE GASTRIC CONTENTS

Stomach digestion consists mainly in the action of pepsin upon proteins in the presence of hydrochloric acid, and in the curdling of milk by rennin.[1] The fat-splitting ferment, lipase, of the gastric juice has very little activity excepting upon previously emulsified fats such as those of milk and egg-yolk.

Pepsin and rennin are secreted by the gastric glands as zymogens—pepsinogen and renninogen respectively—which are converted into pepsin and rennin by hydrochloric acid. Hydrochloric acid is secreted chiefly by the fundus end of the stomach. It at once combines loosely with the proteins of the food, forming acid-metaprotein, the first step in protein digestion. Hydrochloric acid, which is thus loosely combined with proteins, is called "combined" hydrochloric acid. The acid, which is secreted after the proteins present have all been converted into acid-metaprotein, remains as "free" hydrochloric acid and, together with pepsin, continues the process of digestion.

At the height of digestion the stomach contents consist essentially of: (1) water; (2) free hydrochloric acid; (3) combined hydrochloric acid; (4) pepsin; (5) rennin; (6) mineral salts, chiefly acid phosphates of no clinical importance; (7) particles of undigested and partly digested food; (8) various products of digestion in solution. In pathologic conditions there may be present, in addition, various microscopic structures and certain organic acids, of which lactic acid is most important.

Gastric analysis has of late years fallen into some disrepute, probably because too much has been expected of it. The results are influenced by many intra- and extragastric factors, and can be interpreted only in the light of the clinical findings. Excepting in the very rare instances of recovery of good-sized bits of diseased tissue there are no pathognomonic signs.

[1] It is customary to speak of rennin as a separate enzyme, although its independent existence apart from pepsin is unproved.

A **routine examination** is conveniently carried out in the following order. The first two steps must, of course, be modified when the "fractional method" is adopted.

1. Give the patient a test-meal upon an empty stomach, washing the stomach previously if necessary.
2. At the height of digestion, usually in one hour, remove the contents of the stomach with a stomach-tube.
3. Measure and examine macroscopically.
4. Filter. A suction filter is desirable, and may be necessary when much mucus is present.
5. During filtration, examine microscopically and make qualitative tests for—(*a*) free hydrochloric acid; (*b*) lactic acid.
6. When sufficient filtrate is obtained, make quantitative estimations of—(*a*) total acidity; (*b*) free hydrochloric acid; (*c*) combined hydrochloric acid (if necessary).
7. Make whatever additional tests seem desirable, as for blood, pepsin, or rennin.

A. Obtaining the Contents

Gastric juice is secreted continuously, but quantities sufficiently large for examination are often not obtainable from the fasting stomach. In clinical work, therefore, it is desirable to stimulate secretion with food—which is the natural and most efficient stimulus—before attempting to collect the gastric fluid. Different foods stimulate secretion to different degrees, hence for the sake of uniform results certain standard "test-meals" have been adopted.

1. Test-meals.—It is customary to give the test-meal in the morning, since the stomach is most apt to be empty at that time. If it be suspected that the stomach will not be empty, it should be washed out with water the evening before.

(1) **Ewald's test-breakfast** consists of a roll (or two slices of bread), without butter, and two small cups (300 to 400 c.c.) of water, or weak tea without cream or sugar. It should be well masticated. The contents of the stomach are to be removed one hour afterward, counting from the beginning, not the end of the meal. This test-meal has long been used for routine examinations. Its disadvantage is that it introduces, with the bread, a variable amount of lactic acid and numerous yeast-cells. This source of error may be eliminated by substituting a shredded whole-wheat biscuit for the roll. The **shredded wheat test-meal** is now widely

used, and is probably the most satisfactory for general purposes; eight arrowroot cookies may also be used.

(2) **Boas' test-breakfast** consists of a tablespoonful of rolled oats in a quart of water, boiled to one pint, with a pinch of salt added. It should be withdrawn in forty-five minutes to one hour. This meal does not contain lactic acid, and is usually given when the detection of lactic acid is important, as in suspected gastric cancer. The stomach should always be washed with water the evening previous.

(3) **Riegel's test-meal** consists of 400 c.c. of bouillon, a broiled beefsteak (about 150–200 gm.), and 150 gm. of mashed potato. Since it tends to clog the tube, it must be thoroughly masticated.

(4) **Fischer's test-meal** is similar, but probably preferable. It consists of an Ewald breakfast plus about $\frac{1}{4}$ pound lean, finely chopped Hamburger steak, broiled, and lightly seasoned. This and Riegel's may be removed in three to four hours. They give somewhat higher acidity values than the Ewald breakfast.

2. Withdrawal of the Contents.—The gastric fluid is obtained by aspiration through a tube. Stomach-tubes are of two general types. The older type, which was the only one in general use until recently, and is still required in some cases, is a moderately stiff rubber tube about 12 mm. in diameter. It should have an opening at the tip, and one or two in the side near the tip. Near the other end is generally inserted a large bulb for use as an aspirator when necessary. The newer type of stomach-tube, of which the Rehfuss model is most widely used, is a modification of Einhorn's duodenal tube (Fig. 178). It is a very flexible rubber tube about 3 to 4 mm. in diameter with a perforated, olive-shaped, metal tip. A large glass aspirating syringe accompanies the tube.

The use of the former, the Boas tube with bulb, will be first described. It should be sterilized by boiling before and after using.

It is important confidently to assure the patient that introduction of the tube cannot possibly harm him; and that, if he can control the spasm of his throat, he will experience very little choking sensation. When patients are very nervous it is well to spray the throat with cocain solution.

The tube should be dipped in warm water just before using, or chilled in ice-water in order to reduce nausea; the use of glycerin or other lubricant is undesirable. With the patient seated upon a

chair, his clothing protected by towels or a large apron, and his head tilted forward, the tip of the tube, held as one would a pen, is introduced far back into the pharynx. He is then urged to swallow, and the tube is pushed boldly into the esophagus until the colored ring upon it reaches the incisor teeth, thus indicating that the tip is in the stomach. If, now, the patient cough, or strain as if at stool, the contents of the stomach will usually be forced out through the tube. Should it fail, the fluid can generally be pumped out by alternate compression of the tube and the bulb. If unsuccessful at first, the attempt should be repeated with the tube pushed a little further in, or withdrawn a few inches, since the distance to the stomach is not the same in all cases. The tube may become clogged with pieces of food, in which case it must be withdrawn, cleaned, and reintroduced. If, after all efforts, no fluid is obtained, another test-meal should be given and withdrawn after a somewhat shorter period, since, owing to excessive motility, the stomach may empty itself in less than the usual time. Care must be exercised to prevent saliva or vomitus running down the outside of the tube, and mingling with the gastric juice in the basin. As the tube is removed it should be pinched between the fingers so as to save any fluid that may be in it.

A more satisfactory method of withdrawing the fluid with this tube is to remove the bulb-section and attach a Politzer bag with a short section of glass tubing intervening. The fluid may then be aspirated into the bag without danger of admixture of saliva or vomitus.

The stomach-tube must be used with great care, or not at all, in cases of gastric ulcer, aneurysm, uncompensated heart disease, and marked arteriosclerosis. Except in gastric ulcer, the danger lies in the retching produced, and the tube can safely be used if the patient take it easily.

The above procedure is made much easier both for physician and patient by use of one of the newer types of stomach-tube, of which the Rehfuss tube is best known. The metal tip is placed well back in the patient's pharynx, and he is directed to swallow several times in rapid succession with his lips closed and with his tongue forming a groove for the tube, upon which he sucks between swallows. Deep breathing will aid in overcoming nausea. After it has reached the stomach, indicated by the colored ring reaching the

incisor teeth, the heavy tip sinks to the most dependent portion, and as much or as little of the stomach contents as is desired may be drawn off by means of an aspirating syringe. If desired the tube may be stiffened somewhat by threading on a long, loosely-fitting, wire stylet, a method first used by M. E. Jutte, with his duodenal tube. The tube is then introduced in the manner described for the Boas tube. Owing to its small diameter it passes easily. When the tube reaches the stomach the wire is withdrawn. A narrow tube of this type is especially useful for the fractional method of examination described below, as it may be left in place for a long time without serious inconvenience to the patient, particularly if he be induced to read. To prevent its passing on through the pylorus it may be attached to the cheek with adhesive plaster.

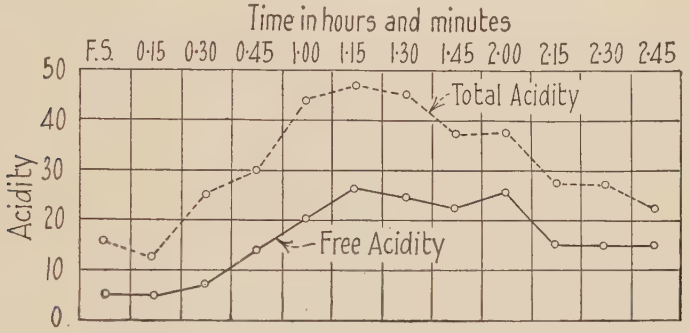


FIG. 174.—Diagram showing the average acidity of stomach fluid of twenty-four healthy persons studied by Talbot by the fractional method: F.S., Fasting stomach.

With the practical appreciation that there is great variation in the time at which the height of digestion is reached, a new method of examination known as the **"fractional method"** has come into wide use. This is carried out as follows:

1. Insert a Rehfuss stomach-tube before breakfast and empty the stomach as far as possible.

2. Remove the tube and give an Ewald test-breakfast, which must be chewed thoroughly.

3. Reinsert the tube and withdraw 5 c.c. of the stomach contents at fifteen-minute intervals, until the fluid is free from food particles or until the acidity has returned to the same level as was found in the fasting content. The tube is left in place during the whole procedure. Ordinarily it causes very little nausea.

4. Examine each of the 5-c.c. portions, and also the fluid from the fasting stomach for total acidity, free hydrochloric acid, and lactic acid.

By means of the Rehfuss tube a much larger quantity of gastric juice can often be obtained from the fasting stomach than was formerly believed possible. The quantity is very variable, ranging from 5 to 150 c.c. or even more, and averaging about 45 c.c. The acidity values are also variable. Averages for the fasting content and each of the fifteen-minute periods are shown in Figure 174. In some normal individuals the height of the acidity curve may be reached earlier; in others ("slowly elaborating stomachs") much later.

Recent work has thrown much doubt upon the value of the fractional method as originally carried out. It has been shown that samples of fluid secured from different parts of the stomach at the same time may differ markedly in acidity, particularly in individuals with gastric disease. Samples obtained in rapid succession through the same tube often differ greatly. It has been suggested that just before each of the fifteen-minute samples is removed the contents be mixed by sucking a portion into the syringe, and forcing it back into the stomach. Probably the best method of fractional analysis is to give a series of test-meals upon successive days, and to remove the entire contents each day at a different period of digestion. In any case only very marked changes in acidity are important.

B. Physical Examination

Under normal conditions 50 to 100 c.c. of fluid can be obtained one hour after administering Ewald's breakfast. Larger amounts point to motor insufficiency or hypersecretion; less than 20 c.c., to too rapid emptying of the stomach, or else to incomplete removal. Upon standing, it separates into two layers: the lower consisting of particles of food; the upper, of an almost clear, faintly yellow fluid. The extent to which digestion has taken place can be roughly judged from the appearance of the food particles.

The **reaction** is frankly acid in health and in nearly all pathologic conditions. It may be neutral or slightly alkaline in some cases of gastric cancer and marked chronic gastritis, or when contaminated by a considerable amount of saliva.

A small amount of **mucus** is present normally. Large amounts, when the gastric contents are obtained with the tube and not vomited, point to chronic gastritis. Mucus is recognized from its characteristic slimy appearance when the fluid is poured from one vessel into another. It is more frequently seen in stomach washings than in the fluid removed after a test-meal.

A trace of **bile** is common as a result of excessive straining while

the tube is in the stomach. Large amounts are very rarely found, and generally point to obstruction in the duodenum. Bile produces a yellowish or more frequently greenish discoloration of the fluid.

Blood is often recognized by simple inspection, but more frequently requires a chemical test for confirmation. It is bright red when very fresh, and dark, resembling coffe-grounds, when older. Vomiting of blood, or *hematemesis*, may be mistaken for pulmonary hemorrhage, or *hemoptysis*. In the former the fluid is acid in reaction, and usually dark red or brown in color, and clotted, while in hemoptysis it is brighter red, frothy, alkaline, and usually mixed with a variable amount of mucus. When the blood is small in amount and bright red, the possibility that it originates from injury done by the tube must not be overlooked.

Particles of food eaten hours or even days previously may be found, and indicate deficient motor power.

Search should always be made for **bits** of **tissue** from the gastric mucous membrane or new growths. These, when examined by a pathologist, will sometimes render the diagnosis clear.

C. Chemical Examination

A routine chemical examination of the gastric contents involves qualitative tests for free hydrochloric acid and organic acids, and quantitative estimations of total acidity, free hydrochloric acid, and sometimes combined hydrochloric acid. Other tests are applied when indicated. In the routine examination qualitative tests are done before quantitative. For this reason all of the qualitative tests are discussed before the quantitative procedures.

1. Qualitative Tests.—(1) **Free Acids.**—The presence or absence of free acids, without reference to the kind, is easily determined by means of Congo-red, although the test is not much used in practice.

Congo-red Test.—Take a few drops of a strong alcoholic solution of Congo-red in a test-tube, dilute with water to a strong red color, and add a few cubic centimeters of filtered gastric juice. The appearance of a *blue color* shows the presence of a free acid (Plate IX, B, B′). Since the test is more sensitive to mineral than to organic acids, a marked reaction points to the presence of free hydrochloric acid.

Thick filter-paper soaked in Congo-red solution, dried, and cut into strips may be used, but the test is much less delicate when thus applied.

(2) **Free Hydrochloric Acid.**—In addition to its digestive function, free hydrochloric acid is an efficient antiseptic. It prevents or retards fermentation and lactic acid formation, and is an important means of protection against the entrance of pathogenic organisms into the body. It is never absent in health. The significance of variations in disease is discussed on page 403.

Dimethylamino-azobenzol Test.—To a little of the filtered gastric juice in a test-tube, or to several drops in a porcelain dish, add a drop of 0.5 per cent. alcoholic solution of dimethylamino-azobenzol. In the presence of free hydrochloric acid there will at once appear a *cherry-red color*, varying in intensity with the amount of acid (Plate X, C). This test is very delicate; but, unfortunately, organic acids, when present in large amounts (above 0.5 per cent.), give a similar reaction. The color obtained with organic acids is, however, more of an orange-red.

Boas' Test.—This test is less delicate than the preceding, but is more reliable, since it reacts only to free hydrochloric acid. It is probably the best routine test.

In a porcelain dish mix a few drops of the gastric juice and the reagent, and slowly evaporate to dryness over a flame, *taking care not to scorch*. The appearance of a *rose-red color*, which fades upon cooling, shows the presence of free hydrochloric acid (Plate IX, 3).

Boas' reagent consists of 5 gm. resublimed resorcinol, and 3 gm. cane-sugar, in 100 c.c. alcohol. The solution keeps well, which, from the practitioner's viewpoint, makes it preferable to Günzburg's phloroglucin-vanillin reagent (phloroglucin, 2 gm.; vanillin, 1 gm.; absolute alcohol, 30 c.c.). The latter is just as delicate, is applied in the same way, and gives a sharper reaction (Plate IX, 4), but is unstable.

(3) **Organic Acids.**—Lactic acid is the most common, and is taken as the type of the organic acids which appear in the stomach contents. It is a product of bacterial activity. Acetic and butyric acids are sometimes present. Their formation is closely connected with that of lactic acid, and they are rarely tested for. When abundant, they may be recognized by their odor upon heating. Butyric acid gives the odor of rancid butter.

Lactic acid is never present at the height of digestion in health. Although often present early in digestion, it disappears when free hydrochloric acid begins to appear. Small amounts may be introduced with the food. Pathologically, small amounts may be present whenever there is stagnation of the gastric contents with

FIG. 1.—A, Uffelmann's reagent; A', A after the addition of gastric fluid containing lactic acid (Boston).

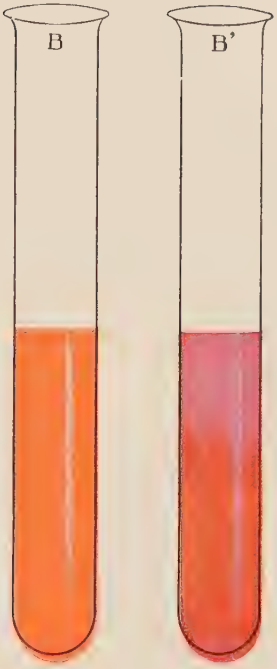


FIG. 2.—B, water to which 3 drops of Congo red solution have been added; B', change induced in B when gastric fluid containing free hydrochloric acid is added (Boston).

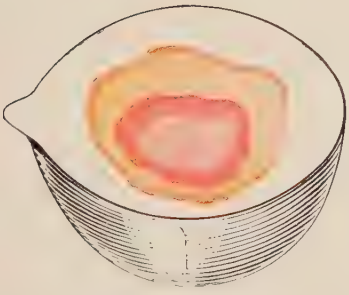


FIG. 3.—Resorcin-test for free hydrochloric acid (Boston).

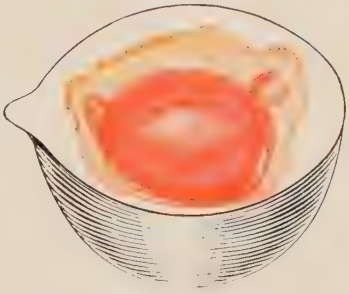


FIG. 4.—Günzburg's test for hydrochloric acid (Boston).

deficient hydrochloric acid, as in many cases of dilatation of the stomach and chronic gastritis. The presence of notable amounts of lactic acid (more than 0.1 per cent. by Strauss' test) is strongly suggestive of gastric cancer, and is probably the most valuable laboratory sign of the disease.

As already stated, the Ewald test-breakfast introduces a small amount of lactic acid, but rarely enough to respond to the tests given here. In every case, however, in which its detection is important, the shredded-wheat biscuit or Boas' test-breakfast should be given, the stomach having been thoroughly washed the evening before.

Uffelmann's Test for Lactic Acid.—Thoroughly shake up 5 c.c. of filtered stomach fluid with 50 c.c. of ether for at least ten minutes. Collect the ether and evaporate. Dissolve the residue in 5 c.c. of water and test with Uffelmann's reagent as follows:

In a test-tube mix 3 drops concentrated solution of phenol and 3 drops saturated aqueous solution of ferric chlorid. Add water until the mixture assumes an amethyst-blue color. To this add the solution to be tested. The appearance of a *canary-yellow* color indicates the presence of lactic acid (Plate IX, A, A′).

Uffelmann's test may be applied directly to the stomach contents without extracting with ether, but is then neither sensitive nor reliable because of the phosphates, sugars, and other interfering substances which may be present.

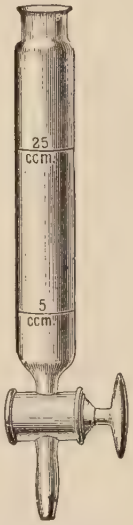


Fig. 175.—Separatory funnel for Strauss' lactic-acid test (Sahli).

Kelling's Test (*Simon's Modification*).—This is much more satisfactory than Uffelmann's. To a test-tube of distilled water add sufficient ferric cholorid solution to give a faint yellowish tinge. Pour half of this into a second test-tube to serve as a control. To the other add a small amount of the gastric juice. Lactic acid gives a distinct yellow color which is readily recognized by comparison with the control. The color is best seen when the tubes are viewed from above over a sheet of paper.

Strauss' Test for Lactic Acid.—This is a good test for clinical work, since it gives a rough idea of the quantity present and is not sufficiently sensitive to respond to the traces of lactic acid which some test-meals introduce. Strauss' instrument (Fig. 175) is essentially a separatory funnel with a mark at 5 c.c. and one at 25 c.c. Fill to the 5-c.c. mark with filtered stomach fluid and to the 25-c.c. mark with ether. Shake thoroughly for ten or fifteen minutes, let stand until the

ether separates, and then, by opening the stop-cock, allow the gastric juice to run out. Fill to the 25-c.c. mark with water and add 2 drops of a 10 per cent. solution of ferric chlorid. Shake gently. If 0.1 per cent. or more lactic acid be present the water will assume a strong greenish-yellow color. A slight tinge will appear with 0.05 per cent.

(4) **Pepsin and Pepsinogen.**—Pepsinogen itself has no digestive power. It is secreted by the gastric glands, and is transformed into pepsin by the action of a free acid. Although pepsin digests proteins best in the presence of free hydrochloric acid, it has a slight digestive activity in the presence of organic or combined hydrochloric acids.

The amount is not influenced by neuroses or circulatory disturbances. Absence or marked diminution, therefore, indicates organic disease of the stomach. This is an important point in diagnosis between functional and organic conditions. Pepsin is rarely or never absent in the presence of free hydrochloric acid.

Test for Pepsin and Pepsinogen.—With a cork-borer cut small cylinders from the coagulated white of an egg, and cut these into disks of uniform size. The egg should be cooked very slowly, preferably over a water-bath, so that the white may be readily digestible. The disks may be preserved in glycerin, but must be washed in water before using.

Place a disk in each of three test-tubes.

Into Tube No. 1 put 10 c.c. distilled water, 5 grains pepsin, U. S. P., and 3 drops of the official dilute hydrochloric acid.

Into Tube No. 2 put 10 c.c. filtered gastric juice.

Into Tube No. 3 put 10 c.c. filtered gastric juice and 3 drops dilute hydrochloric acid.

Place the tubes in an incubator or in warm water for three hours or longer. At intervals observe the extent to which the egg-albumin has been digested. This is recognized by the depth to which the disk has become translucent.

Tube No. 1 is used for comparison, and should show the effect of normal gastric juice.

Digestion of the egg in Tube No. 2 indicates the presence of both pepsin and free hydrochloric acid.

When digestion fails in Tube No. 2 and occurs in No. 3, pepsinogen is present, having been transformed into pepsin by the hydrochloric acid added. Should digestion fail in this tube, both pepsin and pepsinogen are absent.

(5) **Rennin** is the milk-curdling ferment of the gastric juice. It is derived from renninogen through the action of hydrochloric acid. Deficiency of rennin has the same significance as deficiency of pepsin, and is more easily recognized. Indeed, the existence of rennin as a separate enzyme is in doubt, since it has never been isolated. The curdling of milk may, in reality, be due to pepsin.

Test for Rennin.—Neutralize 5 c.c. filtered gastric juice with very dilute sodium hydroxid solution, add 5 c.c. fresh milk, and place in an incubator or in a vessel of water at about 40° C. Coagulation of the milk in ten to fifteen minutes shows a normal amount of rennin. Delayed coagulation denotes a less amount.

(6) **Blood** is present in the vomitus in a great variety of conditions. When found in the fluid removed after a test-meal it commonly points toward ulcer or carcinoma. Blood can be detected in nearly one-half of the cases of gastric cancer. The presence of swallowed blood and blood from injury done by the stomach-tube must be excluded.

Test for Blood in Stomach Contents.—Extract with ether to remove fat if this be present, which is usually not the case after a test-meal. If the fluid be strongly acid, as frequently happens in artificial fluids carelessly prepared for class use, the blood-pigment may go into solution in this ether and be unwittingly discarded.

To 10 c.c. of the fat-free fluid add 3 or 4 c.c. of glacial acetic acid and shake the mixture thoroughly wth about 5 c.c. of ether. Let stand a short time, remove the ether, which forms a layer above the stomach fluid, and use half of it for the guaiac or benzidin test (p. 161). Separation of the ether may be facilitated by adding a small amount of alcohol. In the case of a positive reaction the remainder of the ether-extract may be examined spectroscopically after treating so as to develop the bands of hemochromogen (pp. 324, 326).

When brown particles are present in the fluid the hemin test may be applied directly to them.

2. Quantitative Tests.—(1) **Total Acidity.**—The acid-reacting substances which contribute to the total acidity are free hydrochloric acid, combined hydrochloric acid, acid salts, mostly phosphates, and, in some pathologic conditions, the organic acids. The total acidity is normally about *50* to *75 degrees* (see method below),

or, when estimated as hydrochloric acid, about *0.2* to *0.3 per cent.* With Riegel's or Fischer's test-meal the figures are a little higher.

Töpfer's Method for Total Acidity.—In an evaporating dish or small beaker take 10 c.c. filtered stomach contents and add 3 or 4 drops of the indicator, a 1 per cent. alcoholic solution of phenolphthalein. When the quantity of stomach fluid is small, 5 c.c. may be used, but results are less accurate than with a larger amount. Add decinormal solution of sodium hydroxid drop by drop from a buret, until the fluid assumes a rose-red color which does not become deeper upon addition of another drop (Plate X, A, A′). Most workers accept the first appearance of a permanent pink as the end-point, just as in other titrations with phenolphthalein as indicator; but, owing to interaction of phosphates, Wood advises that the titration of gastric juice be carried a little farther, as here indicated. When this point is reached all the acid has been neutralized. The end reaction will be sharper if the fluid be saturated with sodium chlorid. A sheet of white paper beneath the beaker facilitates recognition of the color change.

In clinical work the amount of acidity is expressed by the number of cubic centimeters of the decinormal sodium hydroxid solution which would be required to neutralize 100 c.c. of the gastric juice, each cubic centimeter representing one *degree* of acidity. Hence, multiply the number of cubic centimeters of decinormal solution required to neutralize the 10 c.c. of stomach fluid by 10. This gives the number of degrees of acidity. The amount may be expressed in terms of hydrochloric acid, if one remembers that each degree is equivalent to 0.00365 per cent. hydrochloric acid. Some one suggests that this is the number of days in the year, the last figure, 5, indicating the number of decimal places.

Example.—Suppose that 7 c.c. of decinormal solution were required to bring about the end reaction in 10 c.c. gastric juice; then $7 \times 10 = 70$ *degrees* of acidity; and, expressed in terms of hydrochloric acid, $70 \times 0.00365 = 0.255$ *per cent.*

Preparation of decinormal solutions is described on page 699. The practitioner will find it best to have them made by a chemist, or to purchase from a chemical supply house.

(2) **Hydrochloric Acid.**—After the Ewald and Boas test-breakfasts the amount of **free hydrochloric acid** varies normally between 25 and 50 degrees, or about 0.1 to 0.2 per cent. In disease it may go considerably higher or may be absent altogether.

When the amount of free hydrochloric acid is normal, organic disease of the stomach probably does not exist.

Increase of free hydrochloric acid above 50 degrees (*hyperchlorhydria*) generally indicates a neurosis, but also occurs in most cases of gastric ulcer and beginning chronic gastritis. It has been found in normal persons suffering from great anxiety or worry.

Decrease of free hydrochloric acid below 25 degrees (*hypochlorhydria*) occurs in some neuroses, chronic gastritis, early carcinoma, pellagra, and most conditions associated with general systemic depression. Marked variation in the amount at successive examinations strongly suggests a neurosis. Too low values are often obtained at the first examination, the patient's dread of the introduction of the tube probably inhibiting secretion.

Absence of free hydrochloric acid (*achlorhydria*) occurs in most cases of gastric cancer and far-advanced chronic gastritis, in many cases of pellagra, and sometimes in hysteria and pulmonary tuberculosis. Achlorhydria is a constant and important symptom of pernicious anemia even during remissions. It sometimes appears long before any anemia is recognizable.

The presence of free hydrochloric acid presupposes a normal amount of **combined hydrochloric acid,** hence the combined need not be estimated when the free acid has been found. When, however, free hydrochloric acid is absent, it is important to know whether any acid is secreted, and an estimation of the combined acid then becomes of great value. The normal average after an Ewald breakfast is about 10 to 15 degrees, the quantity depending upon the amount of protein in the test-meal. Somewhat higher figures are obtained after a Riegel or Fischer test-meal. Of greater significance than the amount of combined acid is the acid deficit, described later.

Töpfer's Method for Free Hydrochloric Acid.—In a beaker take 10 c.c. filtered stomach fluid and add 4 drops of the indicator, a 0.5 per cent. alcoholic solution of dimethylamino-azobenzol. A red color instantly appears if free hydrochloric acid be present. Add decinormal sodium hydroxid solution, drop by drop from a buret, until the last trace of red just disappears, and a canary-yellow color takes its place (Plate X, C, C′). For accuracy it is better (Benedict) not to carry the titration quite to the canary-yellow stage, although the end-point is then not so definite. Read off the number of cubic centimeters of decinormal solution added, and calculate the degrees or percentage of free hydrochloric acid, as in Töpfer's method for total acidity.

When it is impossible to obtain sufficient fluid for all the tests, it will be found convenient to estimate the free hydrochloric acid and total acidity in the same portion, and this is frequently adopted as a routine regardless of the amount of fluid available. After finding the free hydrochloric acid as just described, add 4 drops phenolphthalein solution, and continue the titration. The total amount of decinormal solution used in both the titrations indicates the total acidity.

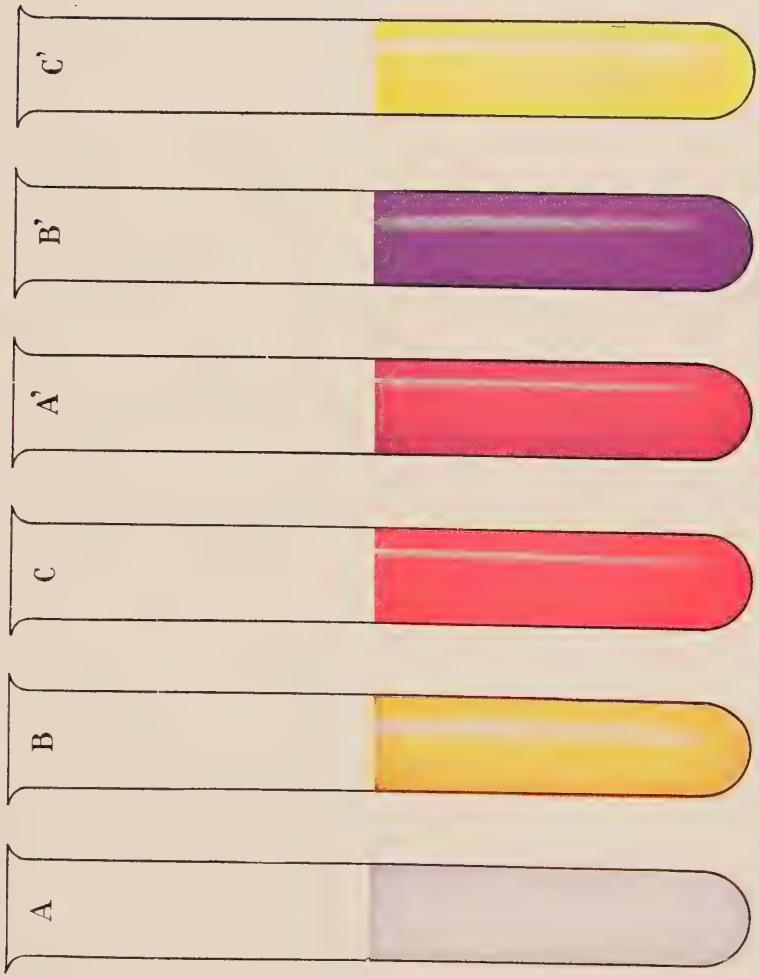
Töpfer's Method for Combined Hydrochloric Acid.—In a beaker take 10 c.c. filtered gastric juice and add 4 drops of the indicator, a 1 per cent. aqueous solution of sodium alizarin sulphonate. Titrate with decinormal sodium hydroxid until the appearance of a violet color which has a slight bluish tinge, and does not become deeper upon addition of another drop (Plate X, B, B′). It is difficult, without practice, to determine when the right color has been reached. It is not always exactly the same, and it is better to watch the color change than to depend upon getting the precise shade. A reddish violet appears first. The shade which denotes the end reaction can be approximately imitated by adding 2 or 3 drops of the indicator to 5 c.c. of 1 per cent. sodium carbonate solution.

Calculate the number of cubic centimeters of decinormal solution which would be required for 100 c.c. of stomach fluid. This gives, in degrees, *all the acidity except the combined hydrochloric acid.* The combined hydrochloric acid is then found by deducting this amount from the total acidity, which has been previously determined.

Example.—Suppose that 5 c.c. of decinormal solution were required to produce the purple color in 10 c.c. gastric juice; then $5 \times 10 = 50 =$ *all the acidity except combined hydrochloric acid.* Suppose, now, that the total acidity has already been found to be 70 degrees; then $70 - 50 = 20$ *degrees* of combined hydrochloric acid; and $20 \times 0.00365 = 0.073$ *per cent.*

When free hydrochloric acid is absent, it is probably more helpful to estimate the **acid deficit** than the combined hydrochloric acid. The acid deficit shows how far the acid secreted by the stomach falls short of saturating the protein (and bases) of the meal. It represents the amount of hydrochloric acid which must be added to the fluid before a test for free hydrochloric acid can be obtained. It is determined by titrating with decinormal hydrochloric acid, using dimethyl-amino-azobenzol as indicator, until the fluid assumes a red color. The amount of deficit is expressed by the number of cubic centimeters of the decinormal solution required for 100 c.c. of the stomach fluid.

PLATE X



A, Gastric fluid to which a 1 per cent. solution of phenolphthalein has been added; B, gastric fluid to which a 1 per cent. solution of alizarin has been added; C, gastric fluid to which a 0.5 per cent. solution of dimethylamino-azobenzol has been added; A', A after titration with a decinormal solution of sodium hydroxid; B', B after titration with a decinormal solution of sodium hydroxid; C', C after titration with a decinormal solution of sodium hydroxid (Boston).

(3) **Organic Acids.**—There is no simple direct quantitative method. After the total acidity has been determined, organic acids may be removed from another portion of the gastric filtrate by shaking thoroughly with an equal volume of neutral ether, allowing the fluids to separate, and repeating this process until the gastric fluid has been extracted with eight or ten times its volume of ether. The total acidity is then determined, and the difference between the two determinations indicates the amount of organic acids.

(4) **Pepsin.**—No direct method is available. The following are sufficient for clinical purposes:

1. **Hammerschlag's Method.**—To the white of an egg add twelve times it volume of 0.4 per cent. hydrochloric acid (dilute hydrochloric acid, U. S. P., 4 c.c.; water, 96 c.c.), mix well, and filter. This gives a 1 per cent. egg-albumen solution. Take 10 c.c. of this solution in each of three tubes or beakers. To *A* add 5 c.c. gastric juice; to *B*, 5 c.c. water with 0.5 gm. pepsin; to *C*, 5 c.c. water only. Place in an incubator for an hour, and then determine the amount of albumin in each mixture by Esbach's method. Tube *C* shows the amount of albumin in the test solution. The difference between *C* and *B* indicates the amount of albumin which would be digested by normal gastric juice. The difference between *C* and *A* gives the albumin which is digested by the fluid under examination. Schütz has shown that the amounts of pepsin in two fluids are proportionate to the squares of the products of digestion. Thus, if the amounts of albumin digested in Tubes *A* and *B* are to each other as 2 is to 4, the amounts of pepsin are to each other as 4 is to 16.

Certain sources of error can be eliminated by diluting the gastric juice several times before testing. The most important of these are that the law of Schütz holds good only for comparatively dilute solutions, and that the products of peptic activity inhibit digestion.

2. **Mett's method** is generally preferred to the preceding. Put three or four Mett's tubes about 2 cm. long into a small beaker with diluted gastric juice (1 c.c. of the filtrate plus 15 c.c. twentieth-normal hydrochloric acid). Place in an incubator for twenty-four hours, and then measure as accurately as possible in millimeters the column which has been digested, using a millimeter scale and a hand-lens or, better, a low power of the microscope and an eye-piece micrometer. Square the average length of this column (because of the law of Schütz mentioned above) and multiply by the degree of dilution, 16. The maximum figure obtained in this way is 256, representing a digested column of 4 mm.

Prepare Mett's tubes as follows: Beat up slightly the whites of one or two eggs and filter. Pour into a wide test-tube, and stand in this a number of capillary glass tubes, 1 or 2 mm. in diameter. When the tubes are filled, plug their ends with bread-crumbs, and coagulate the albumin by heating in water just short of boiling. Dip the ends of the tubes in melted paraffin, and preserve until needed. Bubbles, if present, will probably disappear in a few days. When wanted for use, cut the tubes into lengths of about 2 cm. Discard any in which the albumin has separated from the wall.

D. Microscopic Examination

A drop of unfiltered stomach contents is placed upon a slide, covered with a cover-glass, and examined with the 16-mm. and

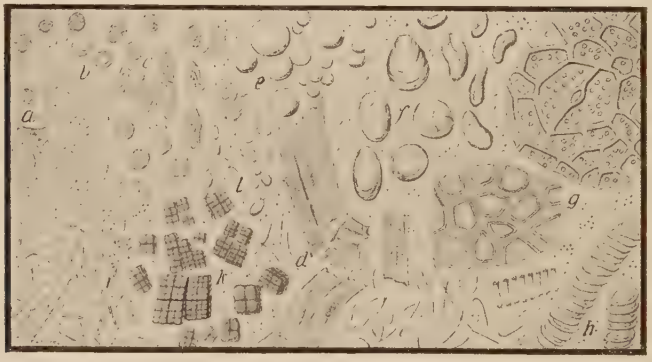


Fig. 176.—General view of the gastric contents: *a*, Squamous epithelial cells from esophagus and mouth; *b*, leukocytes; *c*, cylindric epithelial cells; *d*, muscle-fibers; *e*, fat-droplets and fat-crystals; *f*, starch granules; *g*, chlorophyl-containing vegetable matters; *h*, vegetable spirals; *i*, bacteria, *k*, sarcinæ; *l*, yeast cells (Jakob).

4-mm. objectives with the diaphragm opening much reduced. A drop of greatly diluted Lugol's solution allowed to run under the cover will aid in distinguishing the various structures. As a rule, the microscopic examination is of very limited value.

Under normal conditions little is to be seen except great numbers of starch granules, with an occasional epithelial cell, yeast cell, or bacterium. Starch granules are recognized by their concentric striations, and the fact that they stain blue with iodin solutions when undigested, and reddish, due to erythrodextrin, when partially digested. These colors, however, do not show clearly unless the iodin solution is very dilute.

Pathologically, remnants of food from previous meals, red

blood-corpuscles, pus-cells, sarcinæ, and excessive numbers of yeast cells and bacteria may be encountered (Fig. 176).

Remnants of food from previous meals indicate deficient gastric motility.

Red Blood-corpuscles.—Blood is best recognized by the chemical tests already given. The corpuscles sometimes retain a fairly normal appearance, but are generally so degenerated that only granular pigment is left. When only a few fresh-looking corpuscles are present, they usually come from irritation of the mucous membrane by the tube.

Pus-cells.—Pus is rarely encountered in the fluid removed after a test-meal. Considerable numbers of pus-corpuscles have been found in some cases of gastric cancer. The corpuscles are usually partially digested, so that only the nuclei are seen. The nuclei appear as small highly refractile bodies which lie in clusters of two, three, or four. Swallowed sputum must always be considered.

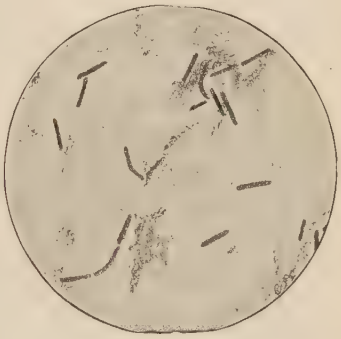


Fig. 177.—Boas-Oppler Bacilli from case of gastric cancer (Boston).

Sarcinæ.—These are small spheres arranged in cuboid groups, often compared to bales of cotton. They frequently form large clumps and are easily recognized. They stain brown with iodin solution. They signify fermentation. Their presence in considerable numbers is some evidence against the existence of gastric cancer, in which disease they rarely occur.

Yeast Cells.—As already stated, a few yeast cells may be found under normal conditions. The presence of considerable numbers is evidence of retention and fermentation. Their appearance has been described (p. 202). They stain yellow to brown with iodin solution.

Bacteria.—Numerous bacteria may be encountered, especially in the absence of free hydrochloric acid. The *Boas-Oppler bacillus* is the only one of special significance. It occurs in the majority of cases of cancer, and is rarely found in other conditions. Carcinoma probably furnishes a favorable medium for its growth. It belongs to the *Bacillus bulgaricus* group.

Boas-Oppler bacilli (Fig. 177) are large (5 to 10 μ long), non-motile, and usually arranged in clumps or end to end in zig-zag chains. They stain yellow to brown with iodin solution, which distinguishes them from *Leptotrichia buccalis* (p. 76) which is not infrequently swallowed, and hence found in stomach fluid. They also stain by Gram's method. They are easily seen with the 4-mm. objective in unstained preparations, but are best recognized with the oil lens, after drying some of the fluid upon a cover-glass, fixing and staining with a simple bacterial stain or by Gram's method.

A few large non-motile bacilli are frequently seen; they should not be reported as Boas-Oppler bacilli unless they are numerous and show something of the typical arrangement.

E. The Gastric Contents in Disease

In the diagnosis of stomach disorders the practitioner must be cautioned against relying too much upon examinations of the stomach contents. A first examination is especially unreliable. Even when repeated examinations are made, the laboratory findings must never be considered apart from the clinical signs.

The more characteristic findings in certain disorders are suggested here:

1. Dilatation of the Stomach.—Evidences of retention and fermentation are the chief characteristics of this condition. Hydrochloric acid is commonly diminished. Pepsin may be normal or slightly diminished. Lactic acid may be detected in small amounts, but is usually absent when the stomach has been washed before giving the test-meal. Both motility and absorptive power are deficient. The microscope commonly shows sarcinæ, bacteria, and great numbers of yeast cells. Remnants of food from previous meals can be detected with the naked eye or microscopically.

2. Gastric Neuroses.—The findings are variable. Successive examinations may show normal, increased, or diminished hydrochloric acid, or even entire absence of the free acid. Pepsin is usually normal.

The presence of more than 100 c.c. of gastric juice in the fasting stomach has until lately been taken to indicate a neurosis characterized by continuous hypersecretion (gastrosuccorrhea), but recent studies of the fasting contents with the Rehfuss tube throw some doubt upon the condition. When the fluid contains food particles, it is the result of retention, not hypersecretion.

3. Chronic Gastritis.—Free hydrochloric acid may be increased in early cases. It is generally diminished in well-marked cases, and is often absent in advanced cases. Lactic acid is often present in traces, rarely in notable amount. Secretion of pepsin and rennin is always diminished in marked cases. Mucus is frequently present, and is very significant of the disease. Motility and absorption are generally deficient. Small fragments of mucous membrane may be found, and when examined by a pathologist may occasionally establish the diagnosis.

4. Achylia Gastrica (Atrophic Gastritis).—This condition may be a terminal stage of chronic gastritis. It is sometimes associated with the blood-picture of pernicious anemia. It gives a great decrease, and sometimes entire absence of hydrochloric acid and ferments. The total acidity may be as low as 1 or 2 degrees. Small amounts of lactic acid may be present. Absorption and motility are not greatly affected.

5. Gastric Carcinoma.—As far as the laboratory examination goes, the cardinal signs are absence of free hydrochloric acid and presence of lactic acid, and of the Boas-Oppler bacillus. These findings are, however, by no means constant, and in any case can be considered only as part of the evidence.

It is probable that some substance is produced by the cancer which neutralizes the free hydrochloric acid, and thus causes it to disappear earlier than in other organic diseases of the stomach. In early cases it may be diminished but slightly or not at all.

The presence of lactic acid is possibly the most suggestive single symptom of gastric cancer. In the great majority of cases its presence in notable amount (0.1 per cent. by Strauss' method) after Boas' breakfast, the stomach having been washed the evening before, warrants a tentative diagnosis of malignancy.

Carcinoma seems to furnish an especially favorable medium for the growth of the Boas-Oppler bacillus, hence this micro-organism is frequently present, and may also be found in the feces in large numbers (p. 434).

Blood can be detected in the stomach fluid by the chemical tests in nearly one-half of the cases, and is more common when the new growth is situated at the pylorus. Blood is present in the stool in nearly every case.

Evidences of retention and fermentation are the rule in pyloric

cancer. Tumor particles are sometimes found late in the disease. Sarcinæ are rarely found.

6. Gastric Ulcer.—There is excess of free hydrochloric acid in about one-half of the cases. In other cases the acid is normal or diminished. Blood is often present in the gastric contents, and is usually, although often intermittently present in the feces as "occult blood." The diagnosis must be based largely upon the clinical symptoms, and where ulcer is strongly suspected, it is probably unwise to use the stomach-tube.

II. ADDITIONAL EXAMINATIONS WHICH GIVE INFORMATION AS TO THE CONDITION OF THE STOMACH

1. Absorptive Power of the Stomach.—This is a very unimportant function, only a few substances being absorbed in the stomach. It is delayed in most organic diseases of the stomach, especially in dilatation and carcinoma, but not in neuroses. The test has little practical value.

Give the patient, upon an empty stomach, a 3-grain capsule of potassium iodid with a glass of water, taking care that none of the drug adheres to the outside of the capsule. At intervals test the saliva for iodids by moistening starch-paper with it and touching with yellow nitric acid. A blue color shows the presence of an iodid, and appears normally in ten to thirty minutes after ingestion of the capsule. A longer time denotes delayed absorption.

Starch-paper is prepared by soaking filter-paper in boiled starch and drying.

2. Motor Power of the Stomach.—This refers to the rapidity with which the stomach passes its contents on into the intestines. It is very important: intestinal digestion can compensate for insufficient or absent stomach digestion only so long as the motor power is good.

Motility is impaired to some extent in chronic gastritis. It is especially deficient in pyloric obstruction caused by malignant or benign new growths; in pyloric spasm, as in hyperchlorhydria; and in atony of the stomach wall which is usually associated with dilatation and gastroptosis.

The best evidence of deficient motor power is the detection of food in the stomach at a time when it should be empty, before

breakfast in the morning. A special test-meal containing easily recognized materials (rice pudding with currants, jam with seeds, or raisins) is sometimes given and removed at the end of six or seven hours. When more than 100 c.c. of fluid are obtained with the tube one hour after an Ewald breakfast, deficient motility may be inferred.

Ewald's salol test is scarcely so reliable as the above. It depends upon the fact that salol is not absorbed until it reaches the intestines and is decomposed by the alkaline intestinal juices.

The patient is given 15 grains of salol with a test-breakfast, and the urine, passed at intervals thereafter, is tested for salicyluric acid. A few drops of 10 per cent. ferric chlorid solution are added to a small quantity of the urine. A violet color denotes the presence of salicyluric acid. It appears normally in sixty to seventy-five minutes after ingestion of the salol. A longer time indicates impaired motor power.

3. To Determine Size and Position of Stomach.—After removing the test-meal, while the tube is still in place, force quick puffs of air into the stomach by compression of the bulb. The puffs can be clearly heard with a stethoscope over the region of the stomach, and nowhere else.

III. DUODENAL CONTENTS

1. Withdrawal of Contents.—The duodenal fluid is obtained by means of a slender, flexible, rubber tube, from 3 to 4 mm. in outside diameter, with a perforated metal tip (Fig. 178). It is marked with a series of black rings to indicate the distances from the incisor teeth to the cardia, to the pylorus, and to the duodenum. The tube was first demonstrated by Einhorn in 1909. There are many modifications, notably those of Jutte and of Rehfuss, which differ chiefly in the shape and weight of the metal tip, and in the arrangement of the performations. The Jutte tube has a wire stylet which facilitates introduction as far as the stomach.

When chief interest centers in the pancreatic ferments it may be well to give a cup of bouillon a half-hour before the tube is introduced, but ordinarily no test-meal is given. The patient abstains from food for about twelve hours, taking only occasional sips of water. The tube is introduced into the stomach in the manner already described for the stomach-tube (p. 394). The patient is

then placed upon his right side with the hips elevated 6 or 8 inches. The movements of the stomach, aided by gravity, carry the metal tip through the pylorus into the duodenum, usually within thirty to forty-five minutes. Fluid should begin to drip from the free end of the tube soon after the metal tip has reached the stomach. If it does not do so, siphonage should be started by injecting a few cubic centimeters of warm water. The fluid is collected in portions of about 5 or 10 c.c. in a series of test-tubes or small bottles. That which first appears is from the stomach, and may be recognized by its acid reaction. When the tube enters the duodenum, the fluid becomes slightly alkaline and is usually clear, light-yellow or colorless, and distinctly viscid. Only in case of gastric anacidity will there be much doubt as to the origin of the fluid. In such cases

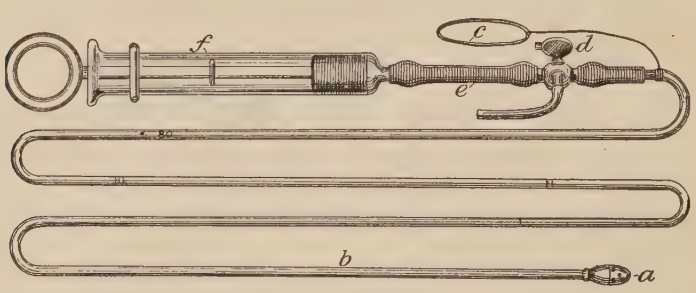


FIG. 178.—Einhorn's duodenal tube: *a*, Perforated metal capsule; *b*, rubber tube with marks at 40, 56, and 70 cm. from the metal capsule; *c*, rubber band which may be placed over ear of patient to hold tube in place; *d*, three-way stop-cock; *e*, collapsible connecting tube; *f*, aspirating syringe. (Kemp.)

Einhorn advises giving the patient a few swallows of milk, and aspirating a portion of the fluid through the tube. If the milk appears, the tube is still in the stomach.

Should the flow be interrupted, injection of a few cubic centimeters of warm water will usually re-establish it. Occasionally, after the tube is in the duodenum, the fluid collected in some of the test-tubes will be somewhat cloudy and opalescent, and less alkaline and less viscid than the usual duodenal fluid. This is due to admixture with stomach fluid which may have passed through the pylorus. Such fluid is to be discarded.

Duodenal fluid should be examined as soon as possible after it is obtained, as many of its characteristics quickly change.

2. Physical Characteristics.—Normally duodenal fluid is clear, colorless or light yellow, distinctly viscid, and slightly al-

kaline to litmus. Admixture of acid gastric juice causes it to become somewhat cloudy and opalescent. Cloudiness due to bacteria and pus corpuscles may be present in inflammation of the duodenum or biliary passages.

3. Chemical Examination.—(1) **Ferments.**—Absence or great diminution of one or all of the pancreatic ferments, amylopsin, steapsin, and trypsin, would indicate deficient pancreatic secretion or occlusion of the pancreatic duct. Their estimation yields much the same information as their estimation in the feces, but is more reliable. Pancreatic fluid from a fistulous tract may show little or no proteolytic activity. The methods which follow are not very accurate, but are useful clinically.[1]

1. **Amylopsin** (**Pancreatic Amylase**).—This ferment should be studied in conjunction with the amylase of the urine (p. 130). Its estimation may be carried out in the same manner as is described for amylase in feces, except that pure duodenal fluid is used instead of the dilute fecal suspension, that 5 c.c. of 1 per cent. starch are employed, and that the volume in each tube is brought to 7 c.c. The amount of amylase is expressed in units, which in this case means the number of cubic centimeters of 1 per cent. starch digested by 1 c.c. of duodenal contents. If, for example, starch digestion is complete in the tube containing 0.2 c.c. of duodenal fluid, the amount of amylase is said to be 25 units. Myers and Fine have found the average to be about 40 units, with extremes of 5 and 200 units in different specimens.

2. **Steapsin** (**Pancreatic Lipase**) (*Myers and Fine*).—Place 1 c.c. of the duodenal fluid in each of two test-tubes and boil one to destroy ferments. To each tube add 1 c.c. neutral ethylbutyrate, 10 c.c. distilled water, and a few drops of toluene. Place in incubator at 38° to 40° C. for twenty-four hours, shaking at intervals. In the presence of lipase the ethylbutyrate will be split and the resulting fatty acid will give an acid reaction in the tube which has not been boiled. Add 2 drops of phenolphthalein indicator to each tube, and titrate to a pink color with twentieth-normal sodium hydroxid. The difference in number of cubic centimeters of the twentieth-normal solution required for the 2 tubes is taken as the index of lipolytic activity. Myers and Fine obtained figures from 0.3 to 4.3 c.c., averaging 1.5 to 2.

3. **Trypsin.**—To about 5 c.c. of the fluid made slightly alkaline with

[1] For more accurate, and also more complicated methods, see McClure, C. W., Wetmore, A. S., and Reynolds, L.: New Methods for Estimating Enzymatic Activities of Duodenal Contents, Arch. Int. Med., vol. 27, p. 706, June, 1921.

weak sodium carbonate solution, if it be not already so, add about a gram of fibrin and a few drops of toluene. Place in the incubator for twenty-four to forty-eight hours. If trypsin be present, the easily recognized products of digestion, tyrosin, and tryptophan can be found. Neutralize the fluid, search for tyrosin crystals with the microscope, and test for tryptophan by adding very dilute bromin-water a drop at a time. The appearance of a reddish-violet color, which quickly disappears if too much bromin is added, denotes the presence of tryptophan.

(2) **Bilirubin.**—There is generally a sufficient amount of unaltered bile-pigment to give the duodenal contents at least a tinge of yellow, and the depth of color is a rough, though useful guide to the amount present, despite the presence of a variable amount of urobilin. The amount may be recorded as +, ++, or +++, indicating a small, moderate, or excessive amount, respectively. Sometimes the fluid is dark yellow, brown, or even chocolate brown. The presence of bile would rule out complete obstruction of the hepatic or common bile-duct. Its absence does not mean that no bile reaches the duodenum at any time.

(3) **Urobilin** is a reduction product of bilirubin. Its nature and significance are discussed on page 424. A small amount of urobilin is present in the duodenal contents normally; but the chromogen, urobilinogen, is found only when urobilin is present in marked excess.

The presence of urobilinogen and an increase of urobilin have the same significance as an increase of these substances in the feces. In each case there are certain theoretic and practical objections, yet in clinical work estimations of urobilin in feces and duodenal contents appear to have about equal value, and constitute our most definite index of the activity of blood destruction. They are extremely valuable in the diagnosis of the hemolytic anemias, and the study of a case of anemia of doubtful origin is not complete without one or the other.

The most satisfactory clinical method for urobilin is that of Wilbur and Addis, which Schneider applies to the duodenal contents as follows:

1. To 10 c.c. of duodenal contents add 10 c.c. of saturated alcoholic solution of zinc acetate. Shake well and filter.

2. To 10 c.c. of the filtrate add 1 c.c. of Ehrlich's reagent (p. 426). Mix and let stand in a dark place for fifteen minutes.

3. Examine with a spectroscope and dilute with 60 per cent. alcohol

until the bands of both urobilin and urobilinogen have disappeared exactly as is described for urobilin in feces (p. 426). Calculate the dilution value for 1000 c.c. of duodenal contents, remembering that the filtrate used represented 5 c.c. of duodenal fluid. If, for example, the urobilin and urobilinogen bands disappeared when the 10 c.c. of filtrate was diluted to 80 c.c. and to 40 c.c. respectively, then the dilution value of 5 c.c. of duodenal fluid is 16 for urobilin and 8 for urobilinogen; and for 1000 c.c. it would be 200 times this, or 3200 and 1600, with a total dilution value of 4800.

Schneider finds the maximum for healthy medical students to be about 1000 dilutions, with urobilinogen never present. In pernicious anemia and hemolytic jaundice urobilinogen is generally present, and the total dilution value usually reaches 3000 to 5000. Giffin, Sanford, and Szlapka found a striking decrease following splenectomy, particularly in pernicious anemia.

Schneider uses these figures in the following formula, which aims to express the relation between blood regeneration and blood destruction in the form of an index number which he designates as the H-H (hematopoietic-hemolytic) Index: $\frac{Z + Y}{6}$ = H-H index, Z representing the total duodenal urobilin-dilution value in thousands, and Y the red corpuscle count in millions. Under normal conditions (urobilin dilutions 1000, red cell count 5,000,000) the index is 1. When hemolysis is very active it will be above 1 unless counterbalanced by very deficient blood formation. When blood regeneration fails the index will be below 1. Such an index is artificial and arbitrary, but is useful in impressing the importance of considering the ratio of blood formation to blood destruction in the course of an anemia.

4. Microscopic Examination.—The duodenal fluid must be examined within a few minutes after it is secured, otherwise the cellular elements may be damaged or destroyed by the ferments. The method is the same as for fresh urine. Normally only an occasional leukocyte or epithelial cell can be found. In pathologic conditions these may be present in increased numbers, but no definite diagnostic inferences can be drawn. A great excess of pus corpuscles would suggest inflammation of the duodenum or biliary tract. *Strongyloides stercoralis* and *Giardia lamblia* have been found, sometimes in great numbers. Cystic and vegetative forms of *Endamœba histolytica* have also been found, and in such cases infection of the liver or bile passages is inferred.

5. Bacteriologic Examination.—At the present time very little of clinical value can be learned from a bacteriologic study. Normally the fluid is sterile or contains only a few Gram-positive cocci. Bacteria seen in the direct microscopic examination are mostly dead.

For bacteriologic examination the duodenal fluid is obtained in the usual way, with the following precautions to prevent contamination[1]:

Sterilize the tube by boiling. Slip over the metal tip a gelatin capsule which has been soaked in alcohol for several days. Dip the gelatin-covered tip in thin shellac several times, letting it dry after each coating. Introduce the tube in the usual way. When it has entered the duodenum, the gelatin bag may be removed by forcing in a little air or a few cubic centimeters of sterile water.

IV. EXAMINATION OF FRESH BILE

Following an observation of Meltzer upon the effect of magnesium sulphate applied locally to the mucosa of the duodenum, Lyon has advocated a procedure which he believes makes it possible to collect and segregate bile from the different parts of the biliary tract; and he holds that cytologic and bacteriologic study of this bile yields information of much value in the differential diagnosis of cholecystitis, cholelithiasis, and choledochitis.

The procedure may be outlined as follows: From 50 to 100 c.c. of sterile 25 per cent. saturated magnesium sulphate are introduced into the duodenum through a duodenal tube, which is left in place. Magnesium sulphate introduced directly into the duodenum appears to relax the sphincter of the common duct and thereby to induce drainage of the entire biliary tract. The duodenal contents are aspirated into a series of sterile bottles. Golden-yellow bile from the common duct appears very soon, and is designated "A." After a few minutes this rather suddenly gives place to a darker, more viscid bile, which is supposed to come from the gall-bladder and is designated "B." This portion usually amounts to from 30 to 75 c.c., and is succeeded by a clear light yellow bile of low specific gravity which is assumed to be freshly secreted bile from the liver, and is designated "C." The various portions are collected sepa-

[1] For details see: MacNeal, W. J., and Chase, A. F.: A Contribution to the Bacteriology of the Duodenum, Arch. Int. Med., vol. 12, p. 178, August, 1913.

rately, and their color, viscosity, turbidity, and general appearance noted, as well as the presence or absence of mucus. They are also examined microscopically, chiefly for pus corpuscles, and culturally for bacteria. The practical value of these examinations is not settled.

McClure[1] and his colleagues use 5 c.c. oleic acid in 45 c.c. warm water in place of magnesium sulphate which they found tended to depress the biliary function of the liver.

[1] McClure, C. W., Mendenhall, W. L., and Huntsinger, M. E.: Studies in Liver Function. IV. A Procedure for the Uniform Stimulation of the Biliary Flow, Boston Med. and Surg. Jour., vol. 193, p. 1052, December, 1925.

CHAPTER V

THE FECES

As commonly practised, an examination of the feces is limited to a search for intestinal parasites or ova. Much of value can, however, be learned from other simple examinations, particularly a careful inspection. Anything approaching a complete analysis is, on the other hand, a waste of time for the clinician.

The normal stool is a mixture of—(*a*) water; (*b*) undigested and indigestible remnants of food, as starch granules, particles of meat, vegetable cells, and fibers; (*c*) digested foods, carried out before absorption can take place; (*d*) products of the digestive tract, as altered bile-pigments, enzymes, mucus; (*e*) products of decomposition, as indol, skatol, fatty, acids, and various gases; (*f*) epithelial cells shed from the wall of the intestinal canal; (*g*) harmless bacteria, which are always present in enormous numbers.

Pathologically, we may find abnormal amounts of normal constituents, blood, pathogenic bacteria, animal parasites and their ova, and biliary and intestinal concretions.

The stool to be examined should be passed into a clean vessel without admixture of urine. The examination should not be delayed more than a few hours, owing to the changes caused by decomposition. The offensive odor can be partially overcome with 5 per cent. phenol or a little formalin No disinfectant should be used when search for amebæ is to be made; the vessel must be warm, and the stool kept warm until examined. For other protozoa a saline cathartic may be given, and the second stool examined. The first stool is usually too solid, and the later ones too greatly diluted. Some prefer to make the search for amebæ in feces obtained in this way, using both the first and second stools. A jar or bottle which is sent to the laboratory nearly full of feces should be opened with great care, otherwise the gases which may have formed may force the fecal material out with a spurt and soil the hands.

I. MACROSCOPIC EXAMINATION

1. Quantity.—The amount varies greatly with diet and other factors. The average is about 100 to 200 gm. in twenty-four hours. It is much larger upon a vegetable diet.

2. Frequency.—One or two stools in twenty-four hours may be considered normal, yet one in three or four days is not uncommon with healthy persons. The individual habit should be considered in every case.

3. Form and Consistence.—Soft, mushy, or liquid stools follow cathartics and accompany diarrhea. Copious, purely serous discharges without fecal matter are significant of Asiatic cholera, although sometimes observed in other conditions. Hard stools accompany constipation. Rounded scybalous masses are common in habitual constipation, and indicate atony of the muscular coat of the colon. Flattened, ribbon-like stools result from some obstruction in the rectum, generally a tumor or a stricture from a healed ulcer, most commonly syphilitic. When bleeding piles are absent, blood-streaks upon such a stool point to carcinoma.

4. Color.—The normal light or dark brown color is due chiefly to urobilin, which is formed from bilirubin by reduction processes in the intestine, largely the result of bacterial activity. The stools of infants are yellow, owing partly to their milk diet and partly to the presence of unchanged bilirubin.

Diet and drugs cause marked changes: Milk, a light yellow color; cocoa and chocolate, dark gray; various fruits, reddish or black; spinach, dark green; large doses of calomel, green, due to biliverdin; iron and bismuth, dark brown or black; hematoxylin, red.

Pathologically the color is important. A golden yellow is generally due to unchanged bilirubin. Green stools are not uncommon, especially in diarrheas of childhood. They are sometimes met in apparently healthy infants, alternating with normal yellow stools, and have little significance unless accompanied by symptoms. The color is due to biliverdin or, sometimes, to chromogenic bacteria. Putty-colored or "acholic" stools occur when bile is deficient, either from obstruction to outflow or from deficient secretion. The color is due less to absence of bile-pigments than to presence of fat. Similar stools, which have a greasy appearance and mainfestly consist largely of fat or its derivatives, are common

in conditions like tuberculous peritonitis, which interfere with absorption of fats, and in pancreatic disease.

Large amounts of blood produce tarry black, usually viscid stools when the source of the hemorrhage is the stomach or upper intestine, and a dark brown to bright red as the source is nearer the rectum. When diarrhea exists the color may be red, even if the source of the blood is high up. Red streaks of blood upon the outside of the stool are due to lesions of rectum or anus. Amounts of blood too small for recognition by simple inspection constitute "occult blood" and require chemical tests.

5. Odor.—Products of decomposition, chiefly indol and skatol, are responsible for the normal offensive odor. The strength of this odor depends largely upon the amount of meat in the diet, and the activity of putrefactive bacteria in the intestine. Upon a vegetable or milk diet the odor is much less. A sour odor due to fatty acids is normal for nursing infants, and is noted in mild diarrheas of older children. In the severe diarrheas of childhood a putrid odor is common. In adults, stools emitting a very foul stench are suggestive of malignant or syphilitic ulceration of the rectum or gangrenous dysentery.

6. Mucus.—Excessive quantities of mucus are easily detected with the naked eye, and signify irritation or inflammation. When the mucus is small in amount and intimately mixed with the stool, the trouble is probably in the small intestine. Larger amounts, not well mixed with fecal matter, indicate inflammation of the large intestine. Stools composed almost wholly of mucus and streaked with blood are the rule in dysentery, ileocolitis, and intussusception.

In the so-called mucous colic or membranous enteritis, shreds and ribbons of altered mucus, sometimes representing complete casts of portions of the bowel, are passed, especially after an enema. In the ordinary formed stool they usually pass unrecognized unless the feces be well mixed with water. These may appear as firm, irregularly segmented strands, suggesting tapeworms. The mucus sometimes takes the form of brown or black jelly-like masses. In some cases it is passed at variable intervals, with colic; in others, with every stool, with only vague pains and discomfort. It is distinguished from inflammatory mucus by absence of pus-corpuscles. The condition is not uncommon and should be more frequently

recognized. It is probably a secretory neurosis, hence the name "membranous enteritis" is inappropriate.

7. Concretions.—Gall-stones should be searched for on several successive days in every case of obscure colicky abdominal pain. Intestinal concretions (enteroliths) are rare. Intestinal sand, consisting of sand-like grains, has been found in neurotic conditions, such as mucous colitis. It attracts less attention than formerly, since in most cases the so-called sand proves to be vegetable matter, such as seeds of berries or bananas or the hard brown grains from about the seeds of pears. After ingestion of considerable amounts of olive oil, nodules of soap and fat often appear in the feces, and may be mistaken by the patient for gall-stones, particularly when the oil has been given for cholelithiasis.

Concretions can be found by breaking up the fecal matter in a sieve (which may be improvised from gauze) while pouring water over it. It must be remembered that gall-stones, if soft, may go to pieces in the bowel. Gall-stones are readily identified by their faceted surfaces. When facets are absent, the stones can be distinguished from other concretions by detecting cholesterol and bile-pigment in them. The stone is broken up and as far as possible dissolved in ether. If now the ether be slowly evaporated in a watch-glass, crystals of cholesterol will separate out. Addition of one-half volume of alcohol to the fluid will cause it to evaporate more slowly with formation of more perfect crystals. The crystals may be identified microscopically by their characteristic form (Fig. 54); by a carmin color at the edges when treated with a drop of concentrated sulphuric acid on the slide; or by a play of colors—blue, red, green, violet—when treated with a drop of Lugol's solution. To extract bile-pigments treat the parts of the stone which have failed to dissolve in ether with chloroform and then with hot alcohol. A yellow color in the chloroform, and a green in the alcohol show the presence of bilirubin and biliverdin respectively.

8. Animal Parasites.—Segments of tapeworms, and the adults of other parasites are often found in the stool. The method of recovering tapeworm heads is described on page 484. The smaller worms are sought as described for concretions, the material caught by the sieve being floated out in clear water and examined in a thin layer in a flat-bottomed dish over a dark background. The search should be preceded by a vermicide and a brisk purge or, when pin-

worms are sought, by a copious enema. A hand lens or a very low power of the microscope may be needed to identify very small worms such as trichinella or the male pin-worm. Patients often mistake vegetable tissue for intestinal parasites, and many times physicians have been known to make similar mistakes. The most frequent sources of confusion are long fibers from poorly masticated celery or "greens," which suggest round worms; cells from orange, which suggest pin-worms; and fibers from banana, which, because of the segmented structure and the presence of oval cells, suggest tapeworms and ova (Fig. 179). Even slight familiarity with the microscopic structure of vegetable tissue will prevent the chagrin of such errors.

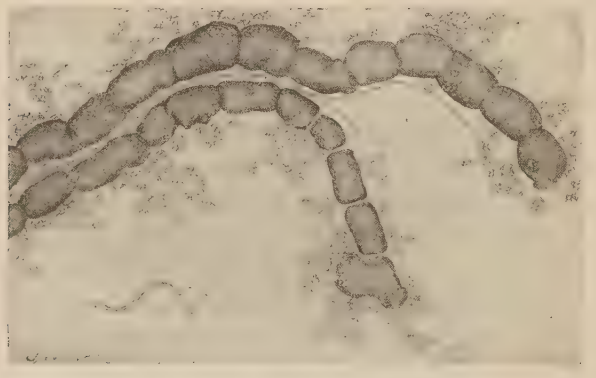


FIG. 179.—Undigested fiber from center of banana in feces (× 15). In the lower part of the figure the fiber is shown natural size. The segments are colored reddish brown when found in the stool. Such fibers in the stools of children with diarrhea are often reported as small tapeworms. In formed stools the segments are often broken apart, and appear as reddish-brown rectangular bodies.

Larvæ of insects are occasionally found, usually the result of accidental contamination, sometimes of swallowing eggs or larvæ. They should be identified as larvæ of insects without difficulty, but the determination of species is a problem for the trained entomologist.

9. Curds.—The stools of infants frequently contain whitish curd-like masses, due either to imperfect digestion of fat or casein, or to excess of these in the diet. When composed of fat, the masses are soluble in ether, and give the sudan III test. If composed of casein, they will become tough and fibrous-like when placed in formalin (10 per cent.) for twenty-four hours.

II. CHEMICAL EXAMINATION

Complicated chemical examinations are of little value to the clinician. Certain tests are, however, important.

1. Reaction.—Normally this is either slightly acid or slightly alkaline. Much depends on the diet; excess of carbohydrates produces acidity, excess of protein, alkalinity. Pathologically, variations in reaction may result from intestinal indigestion of the respective foodstuffs. The reaction may be tested with litmus or with the alizarin indicator used in gastric analysis.

2. Fermentation.—Excessive carbohydrate fermentation, resulting from intestinal indigestion of carbohydrates, is manifested by gas formation and an acid reaction. The stool is usually soft and mushy, and bubbles of gas may be present. The bubbles become much more evident after it has stood in a warm place for twelve hours. Then, the stool, when stirred with a stick, gives a crackling sound resembling râles. As the gas forms the reaction becomes increasingly acid. Normal stool contains few or no gas-bubbles even after standing twenty-four hours. Special diets and special procedures for the determination of gas formation, such as Schmidt's well-known test, may be employed, but are not usually necessary.

Intestinal protein indigestion, on the other hand, is manifested by evidences of putrefaction, that is, very foul-smelling stools with strongly alkaline reaction and little gas formation, together with excess of indican in the urine.

3. Blood.—When present in large amount blood produces such changes in the appearance of the stool that it is not likely to be overlooked. Traces of blood (*occult hemorrhage*) can be detected only by special tests. Recognition of occult hemorrhage has its greatest value in diagnosis of gastric cancer and ulcer. It is constantly present in practically every case of gastric cancer, and is always present, although usually intermittently, in ulcer. Traces of blood also accompany malignant disease of the bowel, the presence of certain intestinal parasites, and other conditions.

Detection of Occult Hemorrhage.—Soften a portion of the stool with water, shake with an equal volume of ether to remove fat, and discard the ether. Treat 10 c.c. of the remaining material with about one-third its volume of glacial acetic acid and extract with about 10 c.c.

ether. Blood-pigment is insoluble in neutral ether, but is readily soluble in acidified ether. Should the ether not separate well, add about one-half its volume of alcohol and mix gently. Apply the guaiac or benzidin test to a portion of the ether as already described (p. 161). When much urobilin is present the color of a positive reaction may be purplish brown. In case the test is positive, it is a good plan to use the remainder of the ether for spectroscopic examination, treating it so as to produce the bands of hemochromogen (pp. 324, 326).

In all cases which give a positive reaction with the patient upon a full diet repeat the test after blood-pigment has been excluded from the food by giving an appropriate diet, bread, milk, eggs, and fruit. At the beginning of the restricted diet give a gram of powdered charcoal or, better, 0.3 gm. of carmin, in capsules, so as to mark the corresponding stool.

Wagner makes a thick smear of the feces on a glass slide or white paper by means of a wooden spatula, allows this to dry, and pours the mixed benzidin reagent on it. The green or blue color is recognized macroscopically and microscopically. Performed in this way the test is not so sensitive, although adequate for all clinical purposes, and diet is not likely to cause confusion unless a great excess of rare or raw meat has been taken.

Roberts' tablets of benzidin and sodium perborate (p. 161) may be used with a concentrated watery suspension of the feces and will be found convenient in office work.

4. Bile.—Normally, unaltered bile-pigment is never present in the feces of adults. In catarrhal conditions of the small intestine bilirubin may be carried through unchanged. It may be demonstrated by the Schmidt test for urobilin, or, if a considerable amount is present, by filtering (after mixing with water if the stool be solid) and testing the filtrate by Gmelin's method, as described under The Urine.

5. Urobilin (Hydrobilirubin).—The urobilin of the urine and the hydrobilirubin which constitutes the principal normal pigment of the feces appear to be identical; and the present tendency is to use the name "urobilin" in both instances. In a general way, the name covers both the pigment, urobilin, and the chromogen, urobilinogen, of which it is an oxidation product, since the two substances have exactly the same significance. For the mode of formation and the significance in the urine the reader is referred to the chapter on The Urine. Owing to constipation and other factors

the amount of urobilin in the feces is subject to marked daily variations. The average of a number of successive daily estimations is, however, fairly constant. Ordinarily the twenty-four-hour stool gives a dilution value by the Wilbur and Addis method of 6000, and 9000 may be taken as the upper normal limit.

Since bilirubin, its mother substance, is a product of blood-pigment, an abnormally large amount of urobilin in the feces or in the duodenal contents may be taken as definite evidence of excessive destruction of red blood-cells within the circulation; and quantitative estimations are of great value whenever such increased blood destruction is in question, even though, as appears to be the case, urobilin excretion does not exactly parallel blood destruction. They have been found especially useful in distinguishing the anemias due to excessive hemolysis (for example, pernicious anemia) from other anemias in which hemolysis is not a prominent factor (carcinoma, hemorrhage); in following the progress of individual cases of pernicious anemia; and in studying the effect of splenectomy performed as a therapeutic measure in this disease. In progressing cases of pernicious anemia the Wilbur and Addis method usually gives urobilin dilution values of 20,000 to 30,000, and often much more; during remissions urobilin may return nearly to the normal. In connection with urobilin excretion the percentage of reticulated red corpuscles in the blood, which serves as a measure of blood regeneration, should be studied, since it is chiefly the balance between blood regeneration and blood destruction which determines the clinical and hematologic pictures in anemia.

Urobilin is nearly or quite absent from the stool in cases of catarrhal jaundice and obstruction of the common or hepatic bile-ducts. Its return will herald relief of the condition before any lessening of the jaundice is evident.

Detection.—The chemical tests mentioned on page 159 may be applied to a watery extract of the stool. Direct spectroscopic examination is impossible owing to the cloudiness of the suspension. The following test is also useful:

Schmidt's Test.—Rub a small quantity of the fecal matter with saturated mercuric chlorid solution and let stand twenty-four hours. Urobilin will give a red color, which is likewise imparted to such microscopic structures as are stained with urobilin. A green color shows the presence of unchanged bilirubin and is not seen normally.

Quantitative Estimation.—The method of Wilbur and Addis is useful clinically. While it does not give the actual quantity of urobilin, it furnishes a rough comparative index which works very well in practice. Because of the instability of urobilin methods which involve elaborate treatment of the feces are not applicable. Since urobilin and urobilinogen have the same significance and are so readily changed one into the other they are included together in the estimation. Estimations are of little value unless the average of six to ten, made on successive days, is taken.

Method of Wilbur and Addis.—1. Collect all the feces for twenty-four hours, keeping them in darkness.

2. Grind the whole quantity with water to a homogeneous paste.

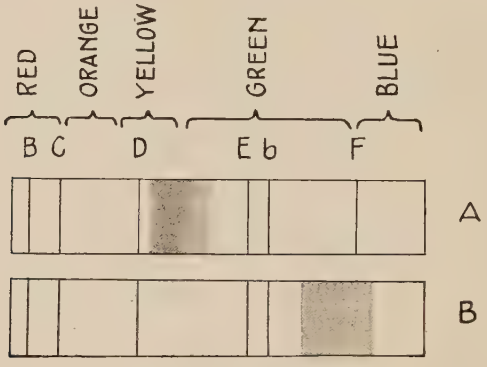


FIG. 180.—Absorption spectra: A, Urobilinogen in acid solution with Ehrlich's reagent; B, urobilin in acid solution with zinc acetate.

3. Dilute to 1000 c.c. with tap-water (or to 500 or 2000 c.c. if the amount of feces is unusually small or large).

4. Measure off 25 c.c. and add to this 75 c.c. acid alcohol (alcohol 64 c.c., concentrated hydrochloric acid 1 c.c., water 32 c.c.).

5. Place in a mechanical shaker for one-half hour. Constant shaking by hand for a similar period will answer.

6. Add 100 c.c. of saturated alcoholic solution of zinc acetate and filter.

7. To 20 c.c. of the filtrate add 2 c.c. of Ehrlich's reagent (para-dimethylaminobenzaldehyd, 20 gm.; concentrated hydrochloric acid, 150 c.c.; water, 150 c.c.).

8. Keep in darkness until next day (or at least for six hours) and examine spectroscopically. In the presence of both urobilinogen and urobilin the two absorption bands indicated in Figure 180, A and B, will be seen.

9. Dilute with 60 per cent. alcohol, adding a few cubic centimeters at a time, until first one and then the other band has entirely disappeared, when the slit of the spectroscope is wide open, but still remains visible when the slit is partly closed. The end-point is fairly definite after one has established his standard upon a series of normal stools. It is perhaps best to use an unvarying width of slit and to dilute until the bands have just disappeared with this opening. One may establish uniform conditions as to the thickness of the layer of fluid, the kind and strength of the light, and the distance from the light, and then adopt a width of slit which gives an average of about 6000 dilutions in a series of normals. When using the "pocket" type of spectroscope we now place the fluid in a standard serologic tube about 12 mm. in diameter and employ a 60-watt frosted Mazda lamp, placed about 6 inches from the spectroscope, which is mounted upon a temporary stand to insure steadiness. The eyes are protected from the light by a cardboard screen.

10. Calculate separately the number of dilutions necessary to cause disappearance of each of the absorption bands and add the two together. The calculation is based not upon the 20 c.c. of filtrate used, but *upon the 2.5 c.c. of fecal suspension represented by the filtrate.* The dilution value for the twenty-four-hour stool (1000 c.c. of fecal suspension) is then found by multiplying this figure by 400. When the fecal suspension was made up to 500 or 2000 c.c. the multiplier would, of course, be 200 or 800. This final result indicates the number of dilutions which would be necessary if all the urobilin and urobilinogen of the twenty-four-hour stool were concentrated in the 2.5 c.c. of fecal suspension examined.

Example.—Suppose that in Step 9 the urobilinogen band disappeared when the 20 c.c. of filtrate had been diluted to 25 c.c., and the urobilin band when the volume reached 30 c.c., then the dilution values for the 2.5 c.c. of feces would be 10 and 12 respectively and the combined value $10 + 12 = 22$. The total dilution value of the twenty-four-hour stool would then be $22 \times 400 = 8800$.

6. Pancreatic Ferments.—Two of the ferments of the pancreatic juice—amylase and trypsin—are normally present in the feces. Lipase can usually not be detected. In pancreatic disease and in simple obstruction of the pancreatic duct these ferments are diminished or absent. Quantitative estimations, therefore, furnish a valuable aid in the diagnosis of pancreatic disease, particularly when carried out in conjunction with an estimation of amylase in the urine. Results, although less reliable, have much the same

significance as those given by examination of the duodenal contents removed through the duodenal tube—a procedure to which the practitioner may sometimes hesitate to resort owing to its technical difficulties and the discomfort to the patient.

Owing to constipation, diet, and other factors there are considerable variations in the amounts of ferments. It is, therefore, essential that a uniform technic be adopted. The following directions are based upon the method recommended by T. R. Brown for amylase. It is best in every case to estimate both amylase and trypsin, but if the examination is limited to one ferment, amylase should be chosen, since the action of trypsin may be simulated by erepsin and the proteolytic activity of bacteria.

Estimation of Pancreatic Ferments in Feces.—1. Upon the evening before the test limit the patient to a light supper and give a high enema at bedtime.

2. At 7.00 next morning give 750 c.c. (25 ounces) of milk.

3. At 7.30 give ½ ounce of Epsom salts; repeat at 8.00.

4. At 8.30 give a glass of water containing ¼ teaspoonful of sodium bicarbonate.

5. Save all the feces passed up to 2 P. M. in a vessel containing 2 ounces of toluol. Keep in a cool place. If less than 400 c.c. are obtained give an enema of 1 pint of water.

6. Dilute the whole volume of feces to 3000 c.c. with normal salt solution, mix well, and centrifugalize a portion for five minutes. Use the supernatant fluid for the following tests:

Estimation of Amylase.—1. Prepare a 1 per cent. solution of soluble starch as follows: To 100 c.c. cold distilled water add 1 gm. soluble starch (Kahlbaum's recommended) and heat gently with constant stirring until clear.

2. Place 2 c.c. of this solution in each of 12 test-tubes.

3. To these tubes add the supernatant fluid from the centrifugalized feces as follows:

To tube 1 add 1.8 c.c.	To tube 7 add 0.6 c.c.
To tube 2 add 1.6 c.c.	To tube 8 add 0.4 c.c.
To tube 3 add 1.4 c.c.	To tube 9 add 0.2 c.c.
To tube 4 add 1.2 c.c.	To tube 10 add 0.1 c.c.
To tube 5 add 1.0 c.c.	To tube 11 add 0.05 c.c.
To tube 6 add 0.8 c.c.	To tube 12 add none (control)

Bring the quantity in each tube up to 4 c.c. with normal salt solution.

4. Place the tubes in an incubator or water-bath at about 38° C.[1] for one-half hour.

5. Fill all tubes with tap-water and add a drop of weak iodin solution to each. Gram's iodin solution will answer.

6. If amylase be present, the series of tubes will vary from yellow through reddish-purple to pure blue, depending upon complete or partial digestion of the starch. The tube before the one in which the first definite trace of blue appears is taken as the measure of digestion. In a series of healthy medical students it was found to be either the ninth or tenth tube, corresponding to 30,000 and 60,000 units[2] respectively.

Test for Trypsin.—The well-known Gross test may be applied as follows:

1. Prepare a 1 : 1000 solution of casein as follows:

Casein, C. P.	0.1 gm.
Sodium bicarbonate	0.1 "
Distilled water	100 c.c.

Boil for one minute, stirring constantly, and cool.

2. Place 5 c.c. of the casein solution in each of 12 test-tubes and add to these tubes the same amounts of the fecal suspension, previously filtered, as were used for the amylase test.

3. Place the tubes in the incubator or a water-bath at 38° C. for one hour.

4. Test for digestion of casein by adding a few drops of 3 per cent. acetic acid to each tube and mixing gently. Digestion is complete in those tubes in which no white precipitate forms, and the tube before the one in which the first definite precipitate appears is taken as the measure of proteolytic activity. In our tests upon healthy medical students this has nearly always been the fourth tube. The end-point is less definite than in the test for amylase.

III. MICROSCOPIC EXAMINATION

Care must be exercised in selection of portions for examination. A random search will often reveal nothing of interest. Samples from several different parts should be examined even when the stool is apparently homogeneous. A small bit of the stool, or any

[1] Variations in reaction and variations in temperature from 37° to 40° C. exert no appreciable effect upon the result.

[2] This means the number of cubic centimeters of 1 per cent. starch solution which would be digested by the 3000 c.c. of fecal suspension under the stated conditions of time and temperature.

suspicious-looking particle, is placed upon a slide, thinned with water if necessary, and covered with a cover-glass. The layer should be just thin enough to read news-print through it when the slide is placed upon the paper. A large slide —about 2 by 3 inches— with a correspondingly large cover will be found convenient. Most of the structures which it is desired to see can be found with a 16-mm. objective. Details of structure must be studied with a higher power Since size is always an important consideration in the identification of microscopic structures, and particularly so in the case of parasites and their ova, frequent use of the eye-piece micrometer is essential. When it is desired to study the food

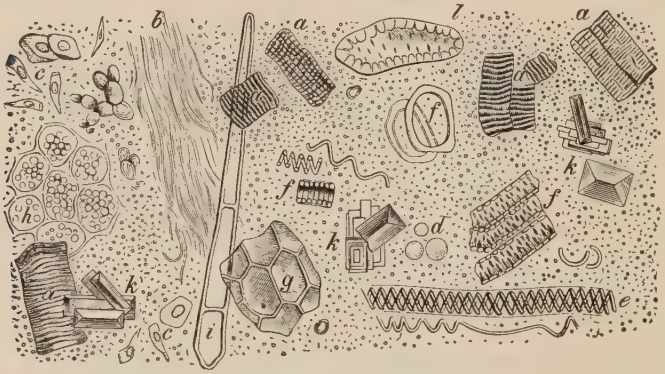


FIG. 181.—Microscopic elements of normal feces: *a*, Muscle-fibers; *b*, connective tissue; *c*, epithelial cells; *d*, white blood-corpuscles; *e*, spiral vessels of plants; *f–h*, vegetable cells; *i*, plant hairs; *k*, triple phosphate crystals; *l*, stone cells. Scattered among these elements are micro-organisms and débris (after v. Jaksch).

remnants as an index of the state of digestion a test-meal should be given and a series of slides prepared as described on page 442.

The bulk of the stool consists of granular débris. Among the recognizable structures (Fig. 181) met in normal and pathologic conditions are: Remnants of food, epithelial cells, pus-corpuscles, red blood-corpuscles, crystals, bacteria, protozoa, and ova of animal parasites.

1. Remnants of Food.—These include a great variety of structures which are very confusing to the student. Considerable study of normal feces is necessary for their recognition.

Vegetable fibers are generally recognized from their spiral structure or their pits, dots, or reticulate markings; **vegetable cells,** from their double contour and the chlorophyl bodies which many

of them contain. These cells are apt to be mistaken for the ova of parasites. **Vegetable hairs** (Fig. 182) frequently look much like the larvæ of some of the worms. Anything like a careful examination will, however, easily distinguish them because of the homogeneous and highly refractile wall, the distinct central canal which extends the whole length, and, especially, the absence of motion. **Starch granules** sometimes retain their original form, but are ordinarily not to be recognized except by their staining reaction. Potato starch appears in colorless translucent masses somewhat like sago grains or flakes of mucus. Starch strikes a blue color with Lugol's solution when undigested; a red color, when slightly digested. **Muscle-fibers** are yellow, and when poorly digested appear as short, transversely striated cylinders with rather squarely broken

FIG. 182.—Vegetable hair (down from skin of peach) in feces (photograph × 150). Compare with Fig. 237.

ends (Fig. 183). Generally the ends are rounded and the striations faint; or only irregularly round or oval yellow masses which bear little resemblance to normal muscle-tissue are found. If a little eosin solution be run under the cover, muscle-fibers will take up the red color and stand out distinctly.

Fats occur in three modifications: neutral fats, fatty acids, and soaps. *Neutral fats* are present in very small amounts or not at all on an ordinary diet. They appear as droplets or yellowish flakes, depending upon the melting-point. They stain strongly with sudan III. *Fatty acids* take the form of flakes like those of neutral fat, or of needle-like crystals which are generally aggregated into thick balls or irregular masses in which the individual crystals are difficult to make out. When treated with sudan III

(p. 669 for formula) the amorphous flakes take a lighter orange than do the neutral fats, while the crystals do not stain. *Soaps*—chiefly calcium soap—appear partly as well-defined yellow amorphous flakes or rounded masses suggesting eggs of parasites, partly as coarse crystals. They do not stain with sudan III and do not melt into globules when warmed as do the fatty acids. **Connective tissue** consists of colorless or yellowish threads with poorly defined edges and indefinite longitudinal striations. When treated with 30 per cent. acetic acid the fibers swell up and become clear and homogeneous. **Elastic fibers,** which are often present along with the connective tissue, are more definite in outline and branch and anastomose. They are rendered more distinct by acetic acid.

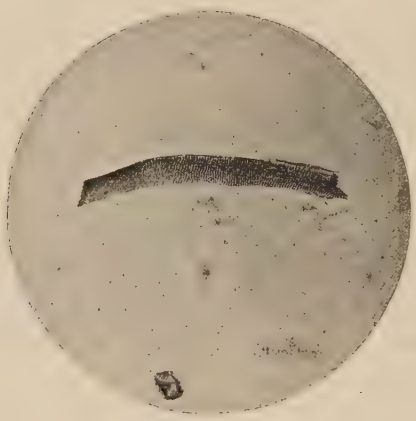


FIG. 183.—Poorly digested muscle-fiber in feces showing striations and ragged ends (photograph × 200).

Excess of any of these structures may result from excessive ingestion or deficient digestion and interpretation should be based upon a standard test diet (p. 441).

2. Body Cells.—A few **epithelial cells,** derived from the wall of the alimentary canal, are always present. They show all stages of disintegration and are often unrecognizable. A marked excess has its origin in a catarrhal inflammation of some part of the bowel, usually the colon when the cells are well preserved. Squamous cells come from the anal orifice, otherwise the form of the cells gives no clue to the location of the lesion.

Pus-corpuscles are present in catarrhal and ulcerative conditions of the intestine. The number of the pus-cells roughly corre-

sponds to the extent and severity of the process except in amebic dysentery, where any considerable number of pus-corpuscles indicates superimposed infection. Amounts of pus sufficient to be recognized with the eye alone indicate rupture of an abscess into the bowel. When the pus is well mixed with the stool the source is high up, but in such cases it is likely to be more or less completely digested and hence unrecognizable.

Haughwout and others have called attention to microscopic findings which they regard as characteristic of bacillary dysentery. The mucus contains large numbers of polymorphonuclear pus-corpuscles, together with a variable number of **macrophages.** These are large mononuclear phagocytic cells with large vesicular nuclei, and frequently contain remnants of ingested leukocytes or red corpuscles. These cells might be mistaken for endamebæ, but the character of the nucleus should make the differentiation easy. They show various degrees of necrosis, and frequently there remains only the circular or oval rim of the cell with a few included granules ("ghost cell"). Haughwout states that "these two types of cells, macrophage and ghost cell, are the two constant and specific characters of the bacillary exudate." In the majority of cases the macrophages are about 2 per cent. of the total number of cells present.

A marked excess of **eosinophils** has been noted in the masses of mucus found in the discharges of intestinal allergy.

Unaltered **red blood-corpuscles** are rarely seen unless their source is the colon, rectum, or anus. A striking tendency of the red cells to form clumps of three or more is mentioned by Anderson as characteristic of amebic dysentery. When the bleeding is in the small intestine the red corpuscles can seldom be recognized as such, and chemical tests must be used (p. 423).

3. Crystals.—Various crystals may be found, but few have any significance. Slender, needle-like crystals of fatty acids and soaps (Fig. 54) and triple phosphate crystals (Fig. 181) are common. Characteristic octahedral crystals of calcium oxalate (Fig. 56) appear after ingestion of certain vegetables. Charcot-Leyden crystals (Fig. 23) are not infrequently encountered, and strongly suggest the presence of intestinal parasites. This may be a helpful point in distinguishing between amebic and bacillary dysentery. Yellowish or brown, needle-like or rhombic crystals of hematoidin (Fig. 54)

may be seen after hemorrhage into the bowel. The dark color of the feces after administration of bismuth salts is due largely to great numbers of bismuth suboxid crystals. They resemble hemin crystals.

4. Bacteria.—In health, bacteria—mostly dead—constitute about one-third of the weight of the dried stool. In general it appears that they are beneficial, although not necessary to existence. Ordinarily it is both difficult and unprofitable to identify them.

Altogether more than fifty different species have been isolated. Some of these are met with only occasionally; some are so constantly present as to be recognized as normal inhabitants of the human intestine. In nursing infants the majority are Gram-positive bacilli of the *Bacillus acidophilus* group, which produce acid, but no gas. This accounts for the normal sour odor of the stools of infants. Most of these disappear soon after the child is weaned, and are replaced by a variety of bacteria, chiefly Gram-negative bacilli of the colon bacillus group, which are the predominant organisms in adult life. A small number of the Gram-positive, acidophilic bacteria generally persists.

For stained preparations the bacteria can be obtained comparatively free from food remnants by mixing a little of the feces with water, allowing to settle for a short time, and making smears from the supernatant fluid. In order to remove fat these films should be fixed by immersion in methyl alcohol for five minutes rather than by heat.

When particles of mucus are found in the fecal discharge they should be washed gently in sterile water, spread on a slide, dried, fixed, and stained. In Asiatic cholera the comma bacillus can often be found in immense numbers in this way.

In some pathologic conditions the character of the intestinal flora changes so that, even in adults, Gram-staining bacteria very greatly predominate. This change is sometimes striking in cases of cancer of the stomach owing to large numbers of Boas-Oppler bacilli, and is of some value in diagnosis. A "Gram-positive stool," due to predominance of cocci, is suggestive of intestinal ulceration.

In cases of excessive intestinal putrefaction it is sometimes possible to check the putrefactive process by substituting for the putrefactive bacteria an acid-producing species such as is normal in the

intestine of nursing infants. For this purpose the Gram-positive *Bacillus acidophilus* is now generally employed. Milk cultures of this organism may be administered, or a food may be given which causes great multiplication of the bacilli of this type which are already present in the intestine. The change from Gram-negative to Gram-positive stool may be observed by counting the proportions of the two types of bacilli in film preparations stained by Gram's method. The technic is the same as when Gram's method is applied to other material (p. 657). Pyronin is a good counterstain. The deep purple Gram-staining bacteria stand out more prominently than the pale red Gram-negative organisms, and one may be misled into thinking them more numerous, even in cases in which they are much in the minority.

Owing to the difficulty of excluding swallowed sputum the presence of the **tubercle bacillus** is less significant in the feces than in other material. It may, however, be taken as evidence of intestinal tuberculosis when clinical signs indicate an intestinal lesion and reasonable care is exercised in regard to the sputum. Success in the search will depend largely upon careful selection of the portion examined. A random search will almost surely fail. Whitish or grayish flakes of mucus or blood-stained or purulent particles should be spread upon slides or covers and stained by the method given on page 200. In the case of rectal ulcers swabs can be made directly from the ulcerated surface. With young children, who swallow all their sputum, an examination of the stool for tubercle bacilli may be the means of diagnosing tuberculosis of the lung.

Bacteria of the **typhoid-dysentery group** are frequently sought as a means of diagnosis of intestinal disorders or as a means of detecting carriers. These bacteria can be found and identified only by cultural methods, which may be carried out as follows:

Mix a small quantity of the feces with about ten times its volume of sterile nutrient broth. Transfer a loopful of the suspension to a large Petri dish containing Endo medium, the surface of which must be dry, and make a series of streaks. Dilute the suspension 1 : 5, 1 : 20, 1 : 100, and 1 : 500, and streak a similar plate from each dilution. In acute dysentery select a particle of blood-stained mucus, wash in sterile water, and streak several plates. It is well to fit a piece of filter-paper into the lid of the Petri dish to absorb moisture. Incubate twelve to twenty-four hours. Colon bacilli give red colonies on this medium and should

be disregarded, although the red will not always appear in a short incubation period. If gray colonies suggesting the typhoid-dysentery group are found, pick off a dozen or more; plant slants of nutrient agar, or better, Russell's double sugar agar, and incubate. Next day study the growth on these tubes, determine motility, make Gram stains, and, if possible, test agglutinability by immune serum (p. 578). This will usually suffice for identification. Should immune serum not be available, plant a series of sugar media, dextrose, lactose, saccharose, maltose, mannite, xylose, containing bromthymol blue as indicator. The cultural characteristics of the typhoid-dysentery group are given on page 669.

5. Yeasts and Molds.—Yeast cells, which show budding and form short chains, are often present in normal stools and may be very numerous in cases of intestinal fermentation. Sarcinæ may be present under normal conditions. Molds are rare and are usually a contamination from unclean vessels or the air. The spores of molds as well as yeast may be mistaken for the cysts of intestinal protozoa.

In most cases of tropical sprue *Monilia psilosis*, a fungus related to the thrush fungus can be isolated from the feces as well as from the lesions on the tongue. It is occasionally found in the stools of healthy persons. Ashford believes that sprue is an infection of *Monilia psilosis* upon a state of nutritional unbalance—chronic digestive incompetence. The organism is best found by making a series of streaks on Petri dishes of Sabouraud's glucose-agar (p. 647). It forms raised, sharply outlined, shiny, creamy white colonies which are most characteristic after several days' growth.[1] Another culture-medium found very useful in isolating this and similar organisms is the tartaric acid medium described on page 647.

A microscopic structure which is frequently found in feces, and the nature and significance of which have not been fully determined, has been described under the name *Blastocystis hominis*. It appears to belong to the yeasts or molds. Lynch[2] has found it in the feces of more than 40 per cent. of hospital patients in Charleston, S. C. It is a colorless, round, sometimes oval body, 5 to 15 μ in diameter,

[1] For details regarding *Monilia psilosis* and sprue see Ashford, B. K.: The Etiology of Sprue, Amer. Jour. Med. Sci., vol. 154, p. 157, August, 1917. Ashford, B. K., and Hernandez, L. G.: Blood-serum Calcium in Sprue and Other Pathologic States in the Tropics, Amer. Jour. Med. Sci., vol. 171, p. 575, April, 1926.

[2] Lynch, K. M.: Blastocystis hominis, Its Characteristics and Its Prevalence in Intestinal Content and Feces in South Carolina, Jour. Bact., vol. 2, p. 369, July, 1917.

or, when actively growing, even 25 μ, and superficially resembles the cysts of some of the intestinal protozoa. It consists of a large central clear body surrounded by a narrow rim of cytoplasm containing a number of refractile spots or nuclei. The whole is surrounded by a delicate capsule (Fig. 184). A case reported by Lynch affords some evidence that it may cause chronic ulceration.

6. Animal Parasites and Ova.—Descriptions will be found in the following chapter. Only general considerations and methods of examination will be given at this place. Frequent use of the eye-piece micrometer is essential, since size is always an important consideration in the identification of parasites and ova.

Of all the **protozoa** which inhabit the bowel of the human being, only two, *Endamœba histolytica* and *Balantidium coli*, are sufficiently harmful to warrant serious clinical consideration.

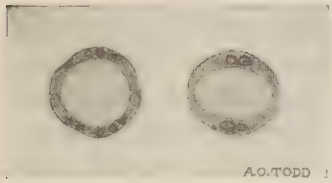


FIG. 184.—*Blastocystis hominis*, a peculiar structure related to the yeasts found in the feces; stained (× 1000) (after Lynch).

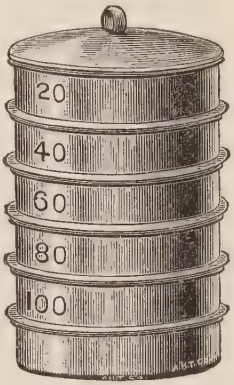


FIG. 185.—Nest of 5-inch sieves of graduated mesh, with cover and receiving pan. These are very useful for fecal examinations. The sieves may be obtained separately.

Spirochetes are found in the feces of approximately half of the persons examined, but have received little attention. They are probably not pathogenic, but may possibly play a secondary rôle in diarrheal conditions. The number is generally small, seldom more than 5 per cent. of the total microbial flora. Their growth is favored by a high protein diet. Many authorities believe the better-known intestinal flagellates, *Giardia*, *Chilomastix*, and *Trichomonas*, to be entirely harmless. There is, however, clinical evidence to indicate that they may cause mild chronic diarrhea or at least aggravate an already existing inflammatory condition. They are rare in carnivorous animals, and Hegner therefore suggests a meat diet in the treatment of infection in man. He reports excellent results in a number of cases.

When protozoa are sought the feces should be obtained without admixture of urine or water. Urine might kill delicate organisms, water might introduce contaminations. In formed stools most protozoa appear in the encysted form, while the actively motile stages, which are more easily recognized by the untrained worker, are ordinarily found only in liquid stools. For this reason it is advisable to give a saline cathartic, and to examine the first liquid stool. Castor oil is not satisfactory because the microscopic droplets of oil are confusing. Most experienced protozoölogists prefer to make the diagnosis from the cysts, and advise against the use of a cathartic. Excepting when endamebæ are in question it is not necessary to keep the stool warm, but it should not be cooled below room temperature. Examination should be made as soon as possible, preferably within a half-hour, since parasitic protozoa in the vegetative or motile stage soon die and disintegrate. They do not encyst after leaving the body. Cysts passed with the feces, on the other hand, remain unchanged for many days.

Not infrequently free-living protozoa are found multiplying in the feces (coprozoic protozoa). This may result from contamination after the feces are passed; or the protozoa may have been swallowed in the encysted stage with the food. Among these are free-living amebæ, which can generally be distinguished from the parasitic species by the presence of a contractile vacuole, and by the continuance of motility for many hours after the stool has cooled. The most important coprozoic flagellates are *Cercomonas* and *Bodo* (p. 470), which were at one time regarded as parasites.

Special Methods for Protozoa.—While the finding of flagellates in feces is generally simple, their accurate identification is difficult. Much depends on the number and position of the flagella, but these are difficult to study in the living specimen. It may be impossible to see them at all until the parasite becomes less active as a result of exposure. Sometimes two or more flagella adhere together, with only the tips free, and thus appear as one. To bring them out more clearly Stitt recommends mixing a drop of Gram's iodin solution with the feces on the slide or, better, use of a dark-ground condenser.

At times, especially in formed stools, no active forms are present and only the cysts can be found. These may be very confusing to the inexperienced. Superficially they resemble yeasts or the spores of molds.

To aid in their detection and differentiation Kofoid strongly recommends a modification of the iodin-eosin method originated by Donaldson:

Eosin, saturated solution in physiologic saline	2 c.c.
Iodin solution (physiologic saline, 100 c.c.; potassium iodid, 5 gm.; iodin to saturation)	1 "
Physiologic saline	2 "

Break up a particle of the feces in a drop of physiologic saline on a slide, place near it a drop of the iodin-eosin solution, and apply a single large cover-glass over both drops. Unstained portions can then be compared with portions showing various degrees of staining. In stained areas the fecal particles, bacteria, and most yeasts are pink, while protozoal cysts gradually take on a yellow to brown color and stand out distinctly, and their nuclei become more clearly defined.

For detection of *Giardia*, *Chilomastix*, and *Trichomonas*, when they cannot be found in the ordinary microscopic examination, Hegner recommends cultures in egg-Locke medium. This is prepared by shaking 1 egg in a flask with glass beads, adding 200 c.c. of Locke's solution (p. 701), heating over a water-bath with stirring for fifteen minutes, and filtering through cotton with suction. The medium is then tubed and sterilized in an autoclave. A bit of feces about the size of a small pea is placed in a tube of this medium and incubated at 37° C. Flagellates in the motile stage should appear in from twenty-four to forty-eight hours.

For detailed study of flagellates and endamebæ protozoölogists now generally depend on stained preparations. Giemsa's stain may be used as described on page 558, or, better, one of the iron hematoxylin methods, of which the following is one of the simplest:

Make thin smears on slides, using a little blood-serum or egg-white if the material is not already albuminous, and while still moist immerse for fifteen minutes in sublimate-alcohol (saturated solution mercuric chlorid in physiologic saline 30 parts, alcohol 2 parts, glacial acetic acid 2 parts), rinse in alcohol, and treat with diluted tincture of iodin for about five minutes to remove the precipitate of mercury. Remove the iodin by soaking in alcohol for about ten minutes. Rinse in water and, while the film is still moist, stain by Mallory's method as follows: Apply 10 per cent. aqueous solution of ferric chlorid for three to five minutes. Drain off excess of fluid and stain for three to five minutes with hematoxylin solution which has been freshly prepared by dissolving a knife-pointful of hematoxylin crystals in 3 or 4 c.c. of water in a test-tube with the aid of heat. Rinse in water, and decolorize and differentiate in a $\frac{1}{4}$ per cent. aqueous solution of ferric chlorid, keeping the slide in motion. Differentiation should be complete in a few seconds to one or

more minutes, and must be watched carefully with the microscope. It should be carried to the point where the nuclei appear properly stained. This is the one difficult step in the whole procedure and requires some experience. Dehydrate with alcohol, clear with xylol, and mount in balsam.

Nearly all of the **intestinal worms** deposit ova which are characteristic of the species and which, if abundant, can be found without difficulty by direct microscopic examination. Ova of *Strongyloides* ordinarily hatch in the intestine and only the larvæ appear in the feces. Ova of *Tænia saginata* for the most part pass out while still enclosed within the segments of the worm, but a variable number from disintegrated segments can usually be found in the feces. Ova, which are likely to be encountered in this country, are shown in Plate XI.

The error of mistaking vegetable cells for the eggs of parasites is needlessly frequent when one can so easily familiarize oneself with the appearance of vegetable cells by study of feces of persons on a mixed diet. When there seems room for doubt, the structure in question is probably not an ovum. Ova of a given species are fairly uniform in size and nearly always typical in appearance, hence measurement and comparison with the plates should make identification easy. Only very rarely need there be any real difficulty, as in the case of a very atypical unfertilized egg of the common round worm (Fig. 225).

Concentration of Ova.—To find ova when scarce they must be concentrated. Stiles advises thoroughly mixing the stool with a quart or more of water, allowing to settle, pouring off the water almost down to the sediment, and repeating the process as long as any matter floats. The final sediment is poured into a conical glass and allowed to settle. Ova will be found in the fine sediment, which can readily be removed with a pipet. Not all kinds of ova sink to the same level. The same end may be accomplished more efficiently and more quickly by means of the centrifuge; but one must learn how long his individual centrifuge requires to throw down the ova while the lighter particles still float. It is usually less than half a minute, and may be as little as ten or fifteen seconds for the last washing. These methods are more satisfactory if the larger particles are first removed by passing the fecal suspension through two or three layers of gauze, or through a succession of sieves with mesh apertures ranging from 10 to 100 to the inch.

The *brine-flotation method of Kofoid and Barber* is especially useful when a large number of examinations must be made. A large fecal sample is thoroughly mixed with about twice its volume of saturated solution of table salt in a paraffined paste-board cup or a small beaker. A lightly compressed circular disk of No. 1 or No. 0 steel-wool about $\frac{1}{8}$ to $\frac{1}{4}$ inch thick is then placed in the cup and pushed to the bottom. This carries down all coarse particles. The fluid is then allowed to stand for one hour, during which time the ova rise to the surface. Finally the surface film is looped off with a wire loop about $\frac{1}{2}$ inch in diameter, placed on a slide, and examined without a cover-glass. The objective should be focussed on the surface of the fluid. We have found this method excellent for ova of nematodes and cysts of endamebæ, although inferior to the more time-consuming centrifugal method previously described. According to McDonald it is unsatisfactory for trematode eggs, which fail to rise to the surface.

Such concentration methods are greatly to be preferred to direct microscopic examination of the stool; not only are the ova concentrated, but they are more easily identified than in untreated feces, since bacteria and débris which would otherwise obscure them have been removed. Other and more complicated methods have been devised, but those just given and Pepper's method for hookworm eggs (p. 508) will probably answer all clinical needs.

IV. FUNCTIONAL TESTS

1. Schmidt's Test Diet.—Much can be learned of the various digestive functions from a microscopic study of the feces, especially when the patient is upon a known diet. For this purpose the standard diet of Schmidt is generally adopted. This consists of:

Morning .....0.5 liter milk and 50 gm. toast.
Forenoon.....0.5 liter porridge, made as follows: 40 gm. oatmeal, 10 gm. butter, 200 c.c. milk, 300 c.c. water, one egg, and salt to taste.
Midday.....125 gm. hamburger steak, with 20 gm. butter, fried so that the interior is quite rare; 250 gm. potato, made by cooking 190 gm. potato with 100 c.c. milk and 10 gm. butter, the whole boiled down to 250 c.c.
Afternoon.....Same as morning.
Evening.....Same as forenoon.

At the beginning of the diet the stool should be marked off with carmin or charcoal (p. 424). One should familiarize himself with the feces of normal persons upon this diet. A portion of the stool about the size of a walnut should be rubbed up with water to a

consistency of thick soup, and examined macroscopically and microscopically. The microscopic examination may be facilitated by preparing four slides: one of the diluted feces untreated; one treated with a drop of dilute Lugol's solution; one with 30 per cent. acetic acid; one with sudan III.

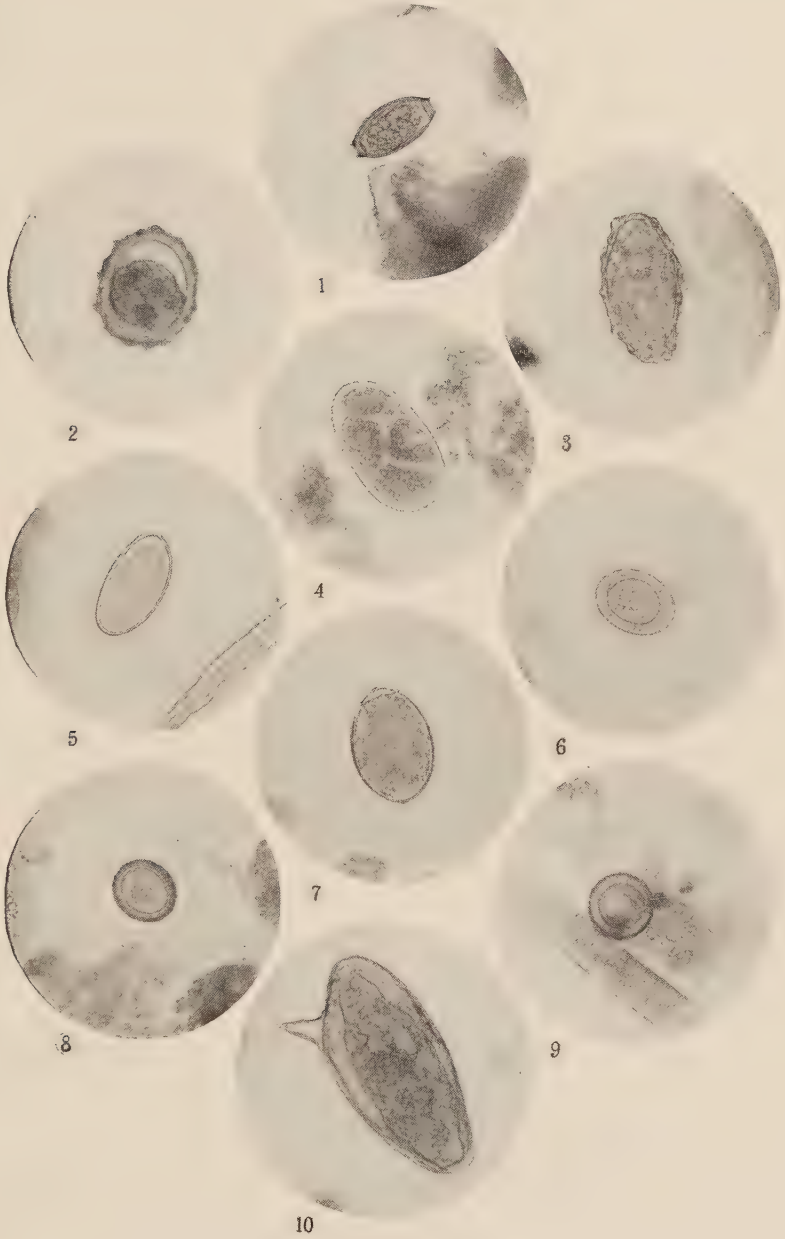
Deficiency of starch digestion is recognized by the number of starch granules which strike a blue color with iodin. With exception of those inclosed in plant cells none are present normally.

The degree of protein digestion is ascertained by the appearance of the muscle-fibers. Striations are clearly visible on any considerable number of the fibers only when digestion is imperfect (Fig. 183). They are most clearly seen in the acetic acid preparation. The striations usually disappear after the feces have stood for some time. According to Schmidt, the presence of nuclei in muscle-fibers denotes complete absence of pancreatic function. The presence of connective-tissue shreds is generally believed to indicate deficient gastric digestion, since it has been accepted that raw connective tissue is digested only in the stomach. Recent work of J. Buckstein, however, indicates that it may to some extent at least be digested in the small intestine. These shreds can be recognized macroscopically by examining in a thin layer against a black background, and microscopically by their fibrous structure and the fact that they swell up and become clear and gelatinous when treated with acetic acid. The only structure likely to cause confusion is elastic tissue, and this is rendered more distinct by acetic acid.

Digestion of fats is checked up by the amount of neutral fat, which should not be present in appreciable quantity normally. It is best seen after staining with sudan III.

Schmidt's nuclei test for pancreatic insufficiency consists in the administration of a 0.5-cm. cube of beef or, better, of thymus, tied in a little gauze bag with the test-meal. The meat must previously have been hardened in alcohol and well washed in water. When the bag appears in the feces it is opened and its contents examined microscopically by pressing out small bits between a slide and cover. A drop of some nuclear stain may be applied if desired. If the nuclei are for the most part undigested, pancreatic insufficiency may be assumed, since it is probable that nuclei can be digested only by the pancreatic juice. Normally the nuclei are digested, provided the time of passage through the intestine is not

PLATE XI



Ova which may be found in the feces, showing comparative size (photographs, × 250): **1,** *Trichuris trichiura* (whip-worm); 2, *Ascaris lumbricoides* (round-worm), fertilized; 3, *Ascaris lumbricoides*, unfertilized; 4, *Necator americanus* (hookworm), four-cell stage; 5, *Oxyuris vermicularis* (pin-worm); 6, *Hymenolepis nana* (dwarf tapeworm); 7, *Diphyllobothrium latum* (fish tapeworm), the edge of the lid being out of focus; 8, *Tænia saginata* (beef tapeworm); 9, *Tænia solium* (pork tapeworm) lying beside a striated muscle-fiber; 10, *Schistosoma mansoni* (blood-fluke).

less than eight or ten hours. Upon the other hand, if the time of passage exceeds thirty hours, nuclei may be partially digested in the complete absence of pancreatic juice.

2. Motility.—Ordinarily, with adults who are upon a mixed diet, twenty to thirty-six hours are required for the passage of ingested material through the gastro-intestinal tract. There is considerable variation among individuals, and usually not all of the residue from a single meal is evacuated at the same time. With infants the time is about one-third as long. In diarrheal conditions it is usually much shortened. In intestinal stasis it may be much prolonged. The time of passage is ascertained by giving 0.5 gm. of powdered charcoal or 0.3 gm. of carmin in a capsule with a meal and watching for the resulting discolored feces. Alvarez and Friedländer gave a capsule containing fifty colored beads about 2 mm. in diameter. On the average about 15 per cent. of the beads were recovered by the end of the first day, 40 per cent. on the second, and 15 per cent. on the third, while the others remained four or five days or even a week. This method has been criticized, on the ground that beads may be delayed by lodging in the stomach or in some pocket of the colon.

CHAPTER VI

ANIMAL PARASITES

Animal parasites are common in all countries, but are especially abundant in tropical and subtropical countries, where, in some localities, almost every native is host for one or more species. Because of our growing intercourse with these regions, and because of our rapidly growing knowledge of parasitology, the subject is assuming increasing importance in this country. Many parasites, hitherto comparatively unknown here, will probably become fairly common.

Some parasites produce no symptoms even when present in large numbers. Others cause very serious symptoms. It is, however, impossible to make a sharp distinction between pathogenic and non-pathogenic species. Parasites which cause no apparent ill effects in one individual may, under certain conditions, produce marked disturbances in another. The disturbances are so varied, and frequently so indefinite, that diagnosis can rarely be made from the clinical symptoms. It must rest on detection, by the naked eye, the microscope, or other means of (*a*) the parasites themselves, (*b*) their ova or larvæ, or (*c*) in a few cases, certain phenomena which depend on the reaction of the body tissues to the parasite, as complement fixation in echinococcus disease.

Unlike bacteria, the great majority of animal parasites multiply by means of alternating and differently formed generations, which require widely different conditions for their development. The exceptions are chiefly among the protozoa. Multiplication of parasites within the same host is thus prevented, as well as direct transmission from man to man. In the case of the hookworm, for example, there is no increase in the number of worms in the host's intestine except through reinfection from the outside. The ova are carried out of the intestine and the young must pass a certain period of development in warm, moist earth before they can again enter the human body and grow to maturity. This also explains the geographic distribution of parasites. The hookworm cannot flourish in cold countries; malaria can prevail only in localities in which the mosquito, *Anopheles*, exists, and then only after the mosquitoes have become infected from a human being.

In general, this alternation of periods of development takes place in one of three ways:

1. The young remain within the original host, but travel to other organs, where they do not reach maturity, but lie quiescent until taken in by a new host. A good example is *Trichinella spiralis.*

2. The young or the ova which subsequently hatch pass out of the host, and either (*a*) go through a simple process of growth and development before entering another host, as is the case with the hookworm, or (*b*) pass through one or more free-living generations, the progeny of which infect new hosts, as is the case with *Strongyloides stercoralis.*

3. The young or ova or certain specialized forms either directly (malarial parasites) or indirectly (tapeworms) reach a second host of different species, where a widely different process of development occurs. The host in which the adult or sexual existence is passed is called the *definitive* or final host; that in which the intermediate or larval stage occurs, the *intermediate* host. Man, for example, is the definitive host for *Tænia saginata,* and the intermediate host for the malarial parasites and *Tænia echinococcus.*

In the case of certain worms in which true alternation of generations is lacking, the ova pass out of the host and undergo a process of maturation before they become infective. Eggs of *Ascaris lumbricoides,* for example, are incapable of hatching for a month after they have left the host.

At this place a few words concerning the classification and nomenclature of living organisms in general will be helpful. Individuals which are alike *in all essential respects* are classed together as a *species.* Closely related species are grouped together to form a *genus;* genera that have certain characteristics in common make up a *family;* families are grouped into *orders;* orders, into *classes;* and classes, finally, into the *branches* or *phyla,* which make up the animal and vegetable *kingdoms.* In some cases these groups are subdivided into intermediate groups—subclasses, subfamilies, and occasionally slight differences warrant subdivision of the species into *varieties.* The name of the family ends in *-idæ,* and that of a subfamily in *-inæ.*

The scientific name of an animal or plant consists of two parts, both Latin or Latinized words, and is printed in italics. The first part is the name of the genus and begins with a capital letter; the

second is the name of the species and begins with a lower-case letter, even when it was originally a proper name. When there are varieties of a species, a third part, the designation of the variety, is appended. The author of the name is sometimes indicated in Roman type immediately after the name of the species. Examples: *Borrelia vincenti*, often abbreviated to *B. vincenti* when the genus name has been used just previously; *Staphylococcus pyogenes albus; Necator americanus*, Stiles.

At the present time there is great confusion in the naming and classification of parasites. Some have been given a very large number of names by different observers, and in many cases different parasites have been described under the same name. The alternation of generations and the marked differences in some cases between male and female have contributed to the confusion, different forms of the same parasite being described as totally unrelated species.

The number of parasites which have been described as occurring in man and the animals is extremely large. Only those which are of medical interest are mentioned here. Some knowledge of their morphology, life histories, and means of dissemination, as well as the methods of diagnosis, is indispensable for the present-day physician. They belong to four phyla—Protozoa, Platyhelminthes, Nemathelminthes, and Arthropoda.

PHYLUM PROTOZOA

These are unicellular organisms, the simplest types of animal life. There is very little differentiation of structure. Each contains at least one, and some several, nuclei. Some contain contractile vacuoles; some have cilia or flagella as special organs of locomotion. They reproduce by division, by budding, or by sporulation. Sometimes there is an alternation of generations, in one of which sexual processes appear, as is the case with the malarial parasites. The protozoa are very numerous, the class Sarcodina alone including no less than 5000 species. Most of the protozoa are microscopic in size; a few are barely visible to the naked eye. The beginning student can gain a general idea of their appearance by examining water (together with a little of the sediment) from the bottom of any pond. Such water usually contains amebæ and considerable variety of ciliated and flagellated forms.

The following is an outline of those protozoa which are of medical interest, together with the classes and subclasses to which they belong

PHYLUM PROTOZOA

CLASS I. **SARCODINA.**—Locomotion by means of pseudopodia.

SUBCLASS **Rhizopoda.**—Pseudopodia form lobose or reticulose processes.

Genus	*Species*
Endamœba.	*E. histolytica.*
	E. coli.
	E. gingivalis.
Dientamœba.	*D. fragilis.*
Endolimax.	*E. nana.*
Iodamœba.	*I. williamsi.*

CLASS II. **MASTIGOPHORA (FLAGELLATA).**—Locomotion by means of flagella.

SUBCLASS **Zoömastigophora.**—Forms in which animal characteristics predominate.

Genus	*Species*
Borrelia.	*B. recurrentis.*
	B. vincenti.
	B. refringens.
	B. (?) *bronchialis.*
Leptospira.	*L. icterohæmorrhagiæ.*
	L. icteroides.
Treponema.	*T. pallidum.*
	T. mucosum.
	T. microdentium.
	T. pertenuis.
Trypanosoma.	*T. gambiense.*
	T. rhodesiense.
	T. cruzi.
	T. lewisi.
Leishmania.	*L. donovani.*
	L. tropica.
	L. infantum.
Trichomonas.	*T. hominis.*
Chilomastix.	*C. mesnili.*
Giardia.	*G. lamblia.*

CLASS III. **SPOROZOA.**—All members parasitic. Propagation by means of spores. No special organs of locomotion.

SUBCLASS **Telosporidia.**—Sporulation ends the life of the individual.

Genus	*Species*
Eimeria.	*E. stiedæ.*
Isospora.	*I. hominis.*
Plasmodium.	*P. vivax.*
	P. malariæ.
	P. falciparum.
Babesia.	*B. bigeminum.*

CLASS IV. **INFUSORIA.**—Locomotion by means of cilia.
SUBCLASS **Ciliata.**—Cilia present throughout life.

Genus	*Species*
Balantidium.	*B. coli.*
	B. minutum.

CLASS SARCODINA

SUBCLASS RHIZOPODA

These are protozoa the body substance of which forms changeable protoplasmic processes, or pseudopodia, for the taking in of food and for locomotion. They possess one or several nuclei. Their usual habitat in the body is the mouth and the intestine, although they are occasionally found in other situations. Only one species, *Endamœba histolytica*, is definitely known to be pathogenic, but some degree of familiarity with a number of others is necessary in order to avoid confusion.

1. Genus Endamœba.—(1) **Endamœba histolytica.**—This organism is found, often in large numbers, in the stools of tropical dysentery, and in the pus and walls of hepatic abscesses associated with dysentery. It has also been found on occasions in duodenal contents, sputum, seminal fluid, urine, synovial fluid, and elsewhere. Infection is more common in this country than was at one time supposed, and is not at all rare in the Northern States, where it generally produces mild chronic dysentery, or, in slight infections, no definite symptoms at all. Records of the Mayo Clinic show cases from all of the Northern States, and from five provinces of Canada. Surveys made at the training camps during the World War indicate about 10 per cent. of infection among the general population.

Like most of the intestinal protozoa, *E. histolytica* is found in the feces in two widely different forms, representing its vegetative and encysted stages of development, respectively.

In the vegetative stage, which is found in acute dysentery, and also in quiescent cases when a liquid stool is obtained by catharsis, the parasite is a grayish or colorless, granular, motile cell, usually between 20 and 40 μ in diameter. There appear to be several races which differ only in size. Within a given race the size of individuals is fairly uniform, so that, in any particular sample of feces, the range in size is much less than is indicated by the above figures. The ectoplasm, seen most clearly as a broad zone at the

ends of the pseudopodia, is homogeneous and refractile. The endoplasm is very granular and contains one small round very indistinct nucleus, and usually one or more digestive vacuoles with red blood-corpuscles when blood is present in the stool. The presence of ingested red blood-corpuscles is an extremely valuable diagnostic point, since these are almost never taken in by the non-pathogenic endamebæ. Fragments of leukocytes and other cells may also be present. There is no contractile vacuole, a fact which serves to distinguish this and the other forms which inhabit the intestine of man from the free-living amebæ which occasionally reach the feces as contaminations.

In cases of bacillary dysentery certain large, phagocytic and vacuolated body-cells (macrophages, p. 433) might be mistaken for endamebæ. Some of these even contain red corpuscles. In general, however, they lack the glassy luster which *E. histolytica* shows. Moreover, the nuclei of body cells are much larger and more distinct.

For a short time after it leaves the body, particularly if kept at body temperature, the endameba exhibits the striking and characteristic *ameboid motion*, constantly changing its shape, or actively moving about by means of distinct pseudopodia. This motion should always be seen to establish the identity of the parasite in its vegetative stage. Even experienced protozoölogists find it difficult or impossible to identify quiescent or dead individuals with certainty. *E. histolytica* is the most active of those found in human feces. When examined on a warm stage, immediately after leaving the body, it is seen to move about on the slide with striking rapidity, usually in a straight line, as if it had a definite objective. This motion is compared by Dobell and O'Connor to that of "a slug going at express speed." Gradually it becomes less active, and no longer moves from place to place, but continues to change its shape by sending out pseudopodia from different parts of its surface with characteristic suddenness. After a time the parasite loses its movement entirely, assumes a spherical form, and dies. When motion is once lost it cannot be reëstablished by warming.

To summarize: the two most important characteristics for the identification of *E. histolytica* in its vegetative stage are its active motility in fresh feces and the presence of ingested red blood-corpuscles.

When the presence of endamebæ is suspected, the stool should be passed into a warm vessel, and kept warm until and during the examination; but special precautions in this regard are not necessary if the material be examined within twenty to thirty minutes. An electrically heated and automatically regulated warm stage is most satisfactory, but a useful warm stage can be improvised from a plate of copper with a hole cut in the center. This is placed upon the stage of the microscope, and one of the projecting ends is heated with a small flame. Endamebæ are most likely to be found in grayish or blood-streaked particles of mucus.

In chronic infections without diarrhea, and in "carriers," the motile form can usually be found only after a cathartic which sweeps the parasites out of the intestine before they have encysted. A saline cathartic is usually used. The first liquid stool is the most favorable, later ones being too dilute. At least four examinations, on different days, should be made before a case is pronounced negative.

If a loopful of feces be mixed with a drop of 1 : 10,000 solution of neutral red in salt solution, the dye will be taken up by the endamebæ, and will render them conspicuous without killing them ("vital staining"). The dye is also taken up by certain flagellates, but never, according to Cutler and Williamson, by *Endamœba coli.*

In clinical diagnosis the study of the living and moving parasite is preferred to any method of staining. For more detailed study, especially as a means of accurately classifying the different endamebæ, protozoölogists prefer smears stained with iron-hematoxylin (p. 439). The structure of the nucleus is the principal criterion (Fig. 186, and the table on p. 454).

In "dysentery carriers," and in chronic cases when the stools are formed and hard, most, or all of the parasites become encysted before leaving the intestine. They first pass through a "precystic" stage in which they become reduced in size, and very sluggish in motion, and lose the enclosed food remnants. In this stage they are practically indistinguishable from the corresponding stage of *Endamœba coli;* and differentiation must depend on vegetative or cystic forms which can usually be found in the same stool. As encystment proceeds, the parasite assumes a spherical form and develops a well-defined wall; the nucleus divides into four.

These cysts should be carefully sought whenever in suspected

cases the vegetative forms are not found. For this purpose the stool need not be kept warm. It should be thinned with water or 5 per cent. formalin, and strained through cheese-cloth or a sieve to remove coarse particles. It is then centrifugalized and the sediment washed several times with water or 5 per cent. formalin to remove the innumerable bacteria which would otherwise cloud the field. The cysts are found without difficulty with the 16-mm. objective, and are then studied in detail with the 4-mm. objective.

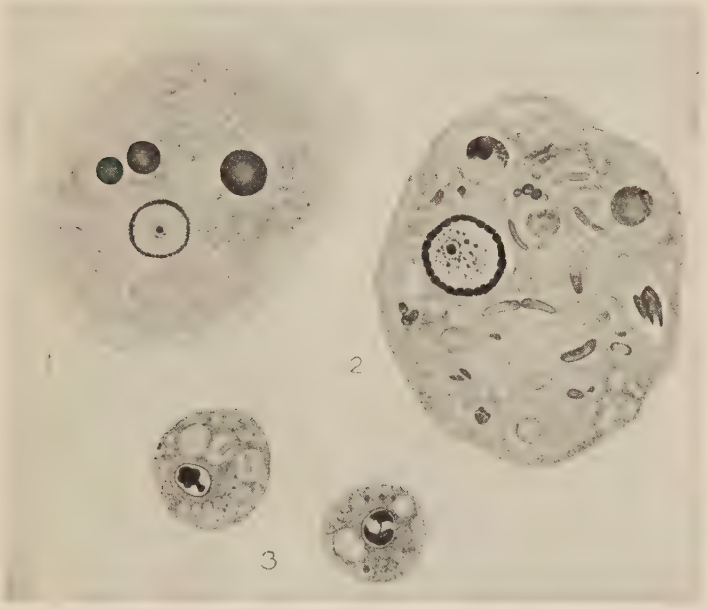


FIG. 186.—The three common intestinal amebæ stained with iron-hematoxylin to show structure of their nuclei as described in the table on page 454. 1. *Endamœba histolytica* with three red blood-cells; 2, *Endamœba coli*, and 3, *Endolimax nana* (× 2000) (After Dobell and O'Connor).

They are colorless, refractile, spheric bodies, 7 to 15 μ in diameter, with a clean-cut edge; and with the low power look not unlike colorless oil-globules. When fully developed they contain four small nuclei. The nuclei lie at different levels and are usually not very distinct, but can generally be made out by careful focusing with the 4-mm. objective and reduced diaphragm opening. Young cysts, with one or two nuclei, contain several highly refractile, colorless granules of chromatin (chromidia), and a small amount of glycogen. A trace of Lugol's solution or of iodin-eosin (p. 439)

added to the fecal material on the slide bring out the structure more clearly.

Encystment is a means of resisting unfavorable conditions, and of dissemination. At this stage alone is the organism infective. Boeck found the maximal length of life of cysts, in water, to be 153 days, and the thermal death-point to be 68° C. The cysts reach a new host chiefly through the food, to which they are carried

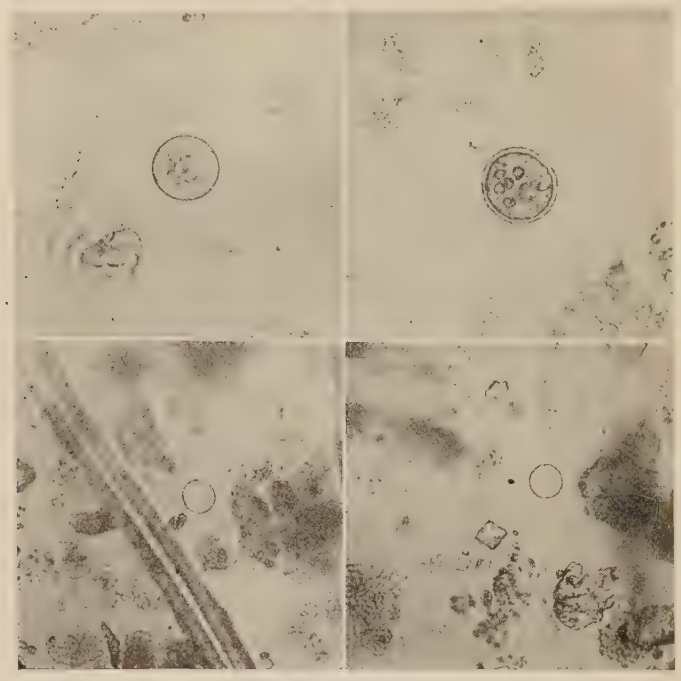


FIG. 187.—Photographs of cysts of *Endamœba coli*. Above, two cysts as seen with oil-immersion objective and low-power eye-piece, one slightly tinged with iodin, one deeply stained with iodin and showing nuclei and double wall (× 525); below, two unstained cysts as seen with 4-mm. objective and low-power eye-piece, one lying beside a vegetable spine, one beside a small calcium oxalate crystal (× 250).

from the feces largely by house-flies. Patients with active dysentery are of little importance in transmitting the disease, since the vegetative forms die, without encystment, soon after leaving the host.

(2) **Endamœba Coli.**—This organism is generally accepted as non-pathogenic, although some authorities believe that it may occasionally take on mildly pathogenic properties when local resistance is lowered. It is found on every continent, and surveys indicate that it is present in the stools of about 20 per cent. of all

persons. While it is thus less frequent than *Endolimax nana*, it has been known for a longer time, and has been more thoroughly studied. In general appearance it resembles *E. histolytica*, but averages somewhat smaller, seldom exceeding 25 μ in diameter. It has, moreover, less distinct pseudopodia, less sharp differentiation between ectoplasm and endoplasm, much less active motion, and a much more distinct nucleus. Its food vacuoles contain bacteria and miscellaneous particles picked up from the feces, but almost never red blood-corpuscles.

Under appropriate conditions the organism forms cysts similar to those of *E. histolytica*. Fully developed cysts (Fig. 187) are 15 to 22 μ in diameter, averaging about 18 μ. They are colorless and highly refractile, with a glassy or, more accurately, a porcelain-like luster, and contain 8 to 16 small nuclei which lie at different levels, and are seen only by careful focusing. The cyst wall is well defined, and usually has a double contour. Their circular shape, sharp outline, and peculiar luster make the search for these cysts with the low power easy.

The principal points of distinction between *E. histolytica* and *E. coli* and *Endolimax nana* are included in the table on pages 454 and 455.

(3) **Endamœba gingivalis.**—That endamebæ are common in the mouth and about the teeth has long been recognized, but they have generally been regarded as harmless or even as beneficial because they feed extensively upon bacteria. There is apparently only one species, which has been variously called *E. buccalis*, *E. dentalis*, and *E. gingivalis*, the last name being now accepted as correct. Within the past few years it has attracted much attention as the possible cause of pyorrhea alveolaris. The organisms are found in the lesions of practically every case of pyorrhea, often in large numbers. In some parts of the slide from which Fig. 188 was made there were as many as 20 in a single field of the oil-immersion lens. Upon the other hand, a few are often found between the gums and teeth when no lesions are recognizable. The evidence at present available suggests that the organism is a factor in the etiology of pyorrhea, but the claim that it is the sole specific cause is not warranted. It is always accompanied by myriads of bacteria, often by "spirochetes," and sometimes by other protozoa, and the rôle which each plays is difficult to determine.

Material is obtained for study by scraping between the teeth and the gum with a sterile wooden toothpick. When pus-pockets exist the bottom and side of a pocket should be scraped with a

CHARACTERISTICS OF THE THREE IMPORTANT INTESTINAL AMEBÆ

VEGETATIVE STAGE

Found in diarrheal stools and in liquid stools following a saline cathartic.

Endamœba histolytica.	*Endamœba coli.*	*Endolimax nana.*
Pathogenic.	Non-pathogenic.	Non-pathogenic.
Size varies with different races of the parasite, between 20 and 40 μ.	Size about the same, 20 to 30 μ.	Very small, 6 to 12 μ, average 8 μ. Important.
Ameboid motion very active in fresh feces. Parasite moves from place to place. Very characteristic.	Motion sluggish. Parasite rarely moves from place to place.	Motion moderately active in very fresh feces, but soon lost.
Ectoplasm hyaline, refractile, sharply differentiated from endoplasm. Characteristic.	Ectoplasm slightly refractile and poorly differentiated from endoplasm.	Ectoplasm and endoplasm not sharply differentiated.
Red blood-cells present in endoplasm when stool contains blood. Practically diagnostic. Few or no ingested bacteria.	Endoplasm contains many bacteria and fecal particles, but very rarely a red blood-cell.	Endoplasm contains bacteria, but no red blood-cells.
Nucleus in living specimen indistinct, often invisible.	Nucleus distinct.	Nucleus indistinct.
Nucleus in specimens stained with iron-hematoxylin shows scanty chromatin arranged in a thin granular ring with a small granule (karyosome) in the center. Diameter 4 to 7 μ (Fig. 186).	Nucleus richer in chromatin and has a thicker peripheral ring of large granules. Karyosome larger and eccentrically placed. One or more granules of chromatin usually present between karyosome and peripheral ring. Diameter 4 to 7 μ (Fig. 186).	Nucleus very small, 1 to 3 μ. Chromatin arranged in several small masses connected by bands (Fig. 186).

Cystic Stage

The cysts are most frequently found in formed stools. If they can be properly studied they offer probably the best means of differentiating the species. They may be studied in the fresh condition with Gram's iodin, or iodin-eosin, or, better, in permanent preparations stained with iron-hematoxylin (p. 439).

Endamœba histolytica.	*Endamœba coli.*	*Endolimax nana.*
Cysts spheric or slightly ovoid. Cyst wall single and delicate in young cysts; thicker and sometimes double outlined in older ones.	Similar, but double outline of wall more frequent and much more distinct. Entire cyst more refractile.	Cyst definitely ovoid, thin-walled.
Size differs with different races of the parasite, 7 to 15 μ.	Usually large, 15 to 22 μ.	Small, 7 to 9 μ.
Cytoplasm of 1- and 2-nucleated cysts granular, often with a poorly defined clear area which stains brown with iodin (glycogen). Presence of chromidia (brightly refractile spindle-shaped or irregular masses of chromatin) characteristic. Glycogen and chromidia disappear as cyst matures.	Similar, but glycogen more abundant and sharply defined when stained with iodin. Chromidia rare.	Glycogen sometimes present as a single mass, staining deeply with iodin. Chromidia absent.
Fully developed cysts contain four small nuclei seen by focusing at different levels.	Fully developed cysts contain eight to sixteen nuclei, eight being the normal number. The four-nucleate stage of developing cyst rarely seen.	Fully developed cysts contain four, very rarely eight, very small nuclei.
With iron-hematoxylin the nuclei, although much smaller, show the same structure as in vegetative stage.	Structure of nuclei same as in vegetative stage.	Structure of nuclei same as in vegetative, but difficult to see owing to their small size.

dental scaler. This material may be examined in the fresh state by mixing it with a little saliva and placing on a warmed slide.

The organism is less active than *E. histolytica*, more so than *E. coli*. Unless motion is seen it will be difficult to recognize. Individuals range in size from 10 to 35 μ. *E. gingivalis* apparently forms cysts very rarely. Those which have been seen resembled cysts of *E. histolytica*.

In general, these endamebæ are more easily identified in stained smears. The smears are made by streaking the toothpick three or four times across the slide. Often one of the streaks will contain many of the parasites and the others only a few. Giemsa's

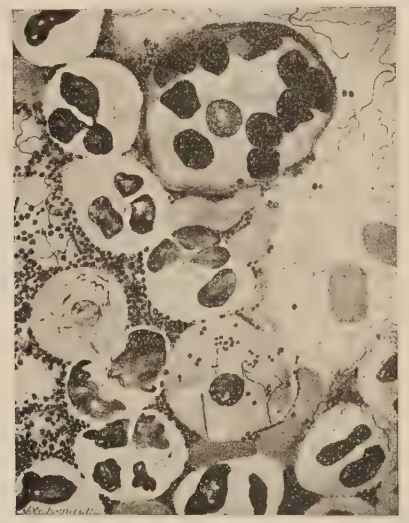


FIG. 188.—*Endamœba gingivalis*, pus-corpuscles, red blood-cells, spirochetes, and bacteria in a smear from a lesion of pyorrhea alveolaris. Giemsa's stain, without alkali, twelve hours (× 850). The figure shows three endamebæ, each with one round nucleus (red). The cytoplasm (deep sky blue) contains vacuoles and bacteria. The largest parasite contains ten nuclei (blackish purple) from ingested cells. A digestion vacuole is seen at each end of the long bacillus in the endameba near the bottom. The red corpuscles were salmon colored; nuclei of leukocytes, reddish purple; spirochetes, bluish purple.

solution, applied as described for blood (p. 275), but allowed to act for three to twelve hours, is the most satisfactory stain. With this the cytoplasm of endamebæ is blue, and shows the vacuoles clearly, the small round nucleus is red, ingested bacteria purple, and nuclei of ingested cells deep purple. In such preparations it is well-nigh impossible to mistake pus and epithelial cells for endamebæ. Wright's stain gives a similar picture, but the differentiation is somewhat less sharp.

2. Genus Endolimax.—*Endolimax nana* is a small endameba

which is now recognized as a frequent inhabitant of the normal colon. Kofoid found it in 28 per cent. of American soldiers. It is 6 to 12 μ in diameter, is sluggishly motile, and never contains red blood-corpuscles. In preparations stained with iron-hematoxylin the nucleus is the most characteristic feature (Fig. 186, and the Table on p. 454). The cysts are ovoid, about 7 to 9 μ in diameter, and usually contain one or two very small faintly visible nuclei, although there are four when fully developed.

3. Genus Iodameba.—*Iodamœba williamsi* resembles very small specimens of *Endamœba coli*. It is about 9 to 13 μ in diameter, is sluggishly motile, and contains a small indistinct nucleus. As a rule, it dies very soon after leaving the intestine. The cysts were known as "iodin cysts" before their nature was recognized. They are spheric, ovoid, or irregular, 9 to 12 μ in diameter, and contain a single nucleus and a large, sharply defined mass of glycogen, which stains dark brown with idoin. Glycogen is usual in the young cysts of most endamebæ, but the bodies are not so large or so sharply defined as in this case, and rarely persist in the mature cyst. Iodameba is harmless and rare.

4. Genus Dientameba.—*Dientamœba fragilis*, also non-pathogenic, is the smallest of the amebæ of the human intestine, usually about 8 μ in diameter. In its vegetative stage it possesses two very small nuclei, is actively motile, and has sharp demarcation between ectoplasm and endoplasm. It is very rare and degenerates quickly after leaving the body. The cysts have been described by Kofoid.

5. Other Endamebæ.—Under the name *Councilmania lafleuri*, Kofoid and Swezy recently described an exceptionally actively motile endameba which ingests red blood-corpuscles and is probably pathogenic. Its cysts are very thick-walled, ovoid, 16 to 20 μ in diameter, and contain eight nuclei. It is not accepted by all protozoölogists.

Recently Brumpt described *Endamœba dispar*, which he claims as a new species found in the feces of man. He states that it has four daughter-cell cysts, does not ingest red blood-cells, and is non-pathogenic for man, but mildly pathogenic for kittens. This ameba is not generally accepted, being considered as a less virulent form of *E. histolytica* found in "carriers."

A number of similar organisms have been described as occurring

in pus, and in ascitic and other body fluids, but it is probable that in many cases, at least, the structures seen were ameboid body cells.

Cultivation of Parasitic Endamebæ.—Boeck and Drbohlav have developed a culture-medium on which parasitic endamebæ may be grown. Four eggs are broken carefully into a sterile flask containing beads, and shaken with 50 c.c. of sterile Locke's solution (p. 701). Slants are made in sterile test-tubes, and inspissated at 70° C. and autoclaved at 15 pounds' pressure for twenty minutes. The slants are then completely covered with 8 parts sterile Locke's solution and 1 part of sterile inactivated human blood-serum. In place of the blood-serum, 10 per cent. ascitic fluid in Locke's solution, or also dilute egg albumin in either Locke's or Ringer's solution, may be used. Dobell has suggested adding a few grains of rice-starch to the fluid part of the medium. The medium is inoculated with a loopful of feces containing endamebæ. Multiplication of these organisms will be found to have taken place in twenty-four to forty-eight hours. This cultural method is useful in diagnosis.

CLASS MASTIGOPHORA (FLAGELLATA)

Subclass Zoömastigophora

The protozoa of this class are provided with one or several whip-like appendages with lashing motion, termed flagella, which serve for locomotion and, in some cases, for feeding. They generally arise from the anterior part of the organism. Some members of the group also possess an undulating membrane—a delicate membranous fold which extends the length of the body and somewhat suggests a fin. When in active motion this gives the impression of a row of cilia. The flagellata do not exhibit ameboid motion, and, in general, maintain an unchanging oval or spindle shape, and contain a single nucleus. The cytoplasm contains numerous granules and, usually, several vacuoles, one or more of which may be contractile. Encystment as a means of resisting unfavorable conditions is common.

Among the most important of the flagellates are the various spiral organisms commonly grouped under the name "spirochetes." These are receiving much attention at the present time, and the appreciation of their medical significance is growing rapidly. Their classification and nomenclature are in an unsettled state. Some authorities place them with the bacteria. Most of the organisms commonly called spirochetes

are now classed with either the genus Borrelia, or Treponema, as the name Spirochæta has been reserved for a different genus of spiral organisms.

Most of them break up into minute granules under certain conditions, each granule being capable of development into a new spirochete. In some cases at least the granules constitute the means of dissemination. Certain spirochetes are transmitted from man to man through the agency of an intermediate host; others by direct contact; still others by the air-borne infective granules.

For the study of the spiral micro-organisms no method is so satisfactory as direct observation of the living parasites by means of dark-ground illumination (p. 23), a method which has been utilized to the fullest extent by Noguchi. In stained preparations they lose many of their distinguishing characteristics, and even their form may be misleading, since they are distorted in the process of spreading the smear. Most of them stain poorly, and are likely to be overlooked in smears stained by the usual methods for bacteria. All, however, are stainable by the methods given for *Treponema pallidum* (p. 558).

1. Genus Borrelia.—(1) **Borrelia recurrentis.**—This "spirochete" was described by Obermeier as the cause of relapsing fever. It appears in the circulating blood during the febrile attack, and, unlike the malarial parasite, lives in the plasma without attacking the red corpuscles. The organism is an actively motile spiral, 15 to 20 μ long, with three to twelve wide, fairly regular turns. It can be seen in fresh unstained blood with a high dry lens, being located by the commotion which it creates among the red cells. For diagnosis thin films, stained with Wright's or some similar blood-stain, are used (Fig. 189). In such preparations the spirals are not so regular.

The organisms of relapsing fever are disseminated by certain ticks and lice which serve as intermediate hosts. In some cases they are transmitted through the eggs to the second or even the third generation of the insect host.

It has been generally believed that relapsing fever does not occur in the United States, but Meader has reported 5 cases which originated in Bear Creek Canon, Colorado, and others have since been recognized in the same locality and in California. Spirochetes from one of these cases are shown in Plate VI.

Besides *Borrelia recurrentis*, a number of distinct strains have

been described in connection with different types of relapsing fever in different localities: *B. novyi*, *B. kochi*, *B. duttoni*, and *B. carteri*.

(2) **Borrelia vincenti.**—In stained smears from the ulcers of Vincent's angina (p. 546) are found what appear to be two organisms. One, the "fusiform bacillus," is a slender rod, 4 to 8 μ long, pointed at both ends, and sometimes curved and often lying in pairs end to end. The other is a slender spiral organism, 10 to 20 μ long, with three to ten comparatively shallow turns (Fig. 272). The staining methods are given on pages 546, 558. These organisms were formerly thought to be bacteria, a spirillum and a bacillus living in symbiosis. The present tendency is to regard them as stages or

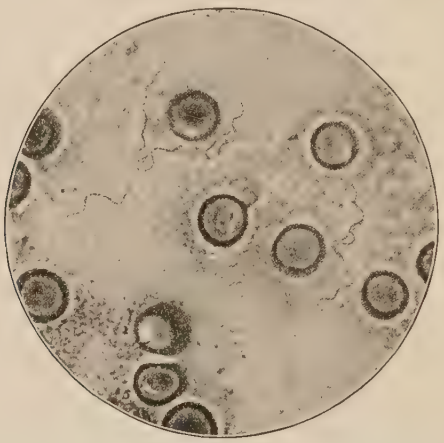


FIG 189.—*Borrelia recurrentis* in the blood of a patient with relapsing fever (× 1000) (Karg and Schmorl).

forms of the same organism, and to class them among the spirochetes, but the question remains unsettled. The same organisms are quite constantly present in large numbers in ulcerative stomatitis, in noma, in erosive and gangrenous balanitis, and in certain leg ulcers in the tropics. Swabs from the surface of the lesions often show many other microörganisms; deeper swabs generally show the spirochete, and the bacillus to dominate the picture. Unless both are present in considerable numbers they cannot be regarded as of etiologic significance. They are not infrequently found in small numbers in normal mouths.

(3) **Borrelia(?) bronchialis.**—The presence in the sputum of this organism which has been called *Spirochæta bronchialis*, but

which probably belongs to the genus *Borrelia*, has been discussed on page 76. It varies strikingly in length, individuals ranging from 5 to 25 μ, depending largely upon the stage of development. It is actively motile in very fresh material, but its motion ceases soon after removal from the body, and the spirochete breaks up into granules which are believed to be the infective forms. There appears to be no intermediate host.

2. Genus Leptospira.—This genus, recently created by Noguchi, includes the spirochetes of infective jaundice and yellow fever.

A form of "rat-bite fever," especially common in Japan, is now known to be caused by a spirochete which probably belongs to this genus.

(1) **Leptospira icterohæmorrhagiæ** is present in various organs, particularly the liver and kidneys, of persons suffering from infectious jaundice or Weil's disease. It averages 6 to 9 μ long, although there is considerable variation among individuals. There appear to be from two to five broad waves, but when examined closely, particularly by dark-ground illumination, its length is seen to be made up of a series of closely set coils. The disease is probably disseminated by rats, for the parasite has been found in the kidneys and urine of apparently healthy rats in many parts of the world, including the United States. The spirochetes are present in the blood early in the disease and may sometimes be found in blood-films, although they are too scarce to make the method of value in diagnosis. They also appear in the urine where they may be very abundant during the third week, and can be found by centrifugalizing thoroughly at high speed and staining the sediment with any good spirochete stain. Since other spiral organisms are frequently present in the urine, one would have to be thoroughly familiar with the morphology of the spirochetes to make the diagnosis in this way. The most reliable means of diagnosis is inoculation of a light-skinned guinea-pig intraperitoneally with 5 c.c. of the patient's blood or urinary sediment. The animal usually dies in about ten days. Autopsy shows jaundice of the skin and widespread small hemorrhages into the tissues and organs.

A form of infectious jaundice, which is probably not of spirochetal origin, is more frequent in the United States.

(2) **Leptospira icteroides** is the spiral micro-organism recently

found by Noguchi in the blood of yellow fever patients, and now thought to be the cause of the disease. It is extremely small and delicate, and can be studied satisfactorily only by dark-ground illumination. Its detection is not of value as a means of clinical diagnosis.

3. Genus Treponema.—(1) **Treponema pallidum.**—This is the organism of syphilis. Its description and methods of diagnosis will be found on pages 556–560.

(2) **Treponema pertenue,** morphologically very similar to *Treponema pallidum,* was found by Castellani in yaws, a skin disease of the tropics.

4. Other "Spirochetes."—A number of harmless forms are of interest because of the possibility of confusing them with the more important pathogenic varieties. Of these, *Treponema mucosum*

FIG. 190.—Spiral organisms: A, *Treponema pallidum;* B, *Borrelia refringens;* C, *Treponema microdentium.* Two red corpuscles are also shown (× 1200).

and *Treponema microdentium* are inhabitants of the normal mouth. When the teeth and gums are not in good condition they are often found in immense numbers (Fig. 188). The former is similar in morphology to *Borrelia vincenti. T. microdentium* (Fig. 190) is smaller (4 to 10 μ), more delicate, has deep curves, and may be easily mistaken for *Treponema pallidum.* It also stains reddish with Giemsa's stain. In suspected syphilitic sores of the mouth it is, therefore, important to make smears from the tissue juices rather than from the surface (p. 558). *Borrelia refringens* is frequently present upon the surface of ulcers, especially about the genitals, and has doubtless many times been mistaken for *Treponema pallidum.* It can be avoided by properly securing the material for examination, but its morphology should be sufficient to prevent confusion. It is thicker than the organism of syphilis, stains more deeply, and has fewer and shallower curves (Figs. 190 and 278). Giemsa's stain

gives it a bluish color. Spiral organisms of various kinds are present in the feces of many normal persons (p. 437).

5. Genus Trypanosoma.—Trypanosomes have been found in the blood-plasma of a great variety of vertebrates. Many of them appear to produce no symptoms, but a few are of great pathogenic importance. They have been much studied in recent years and many species have been described. The forms found in the blood are easily recognized as trypanosomes, but accurate determination of species is difficult and may be impossible from morphology alone. They are elongated, spindle-shaped bodies, the average length of different species varying from 10 to 70 μ. Along one side there runs a delicate undulating membrane, the free edge of which appears to be somewhat longer than the attached edge, thus throwing it into folds. Somewhere in the body, usually near the middle, is a comparatively pale-staining nucleus; and near the posterior end is a smaller, more deeply staining chromatin mass, the micronucleus or blepharoplast. A number of coarse, deeply staining granules, chromatophores, may be scattered through the cytoplasm. A flagellum arises in the blepharoplast, passes along the free edge of the undulating membrane, and is continued posteriorly as a free flagellum. These details of structure are well shown in Plate VI.

The life-history of the trypanosomes is complicated and has not yet been worked out in all details. There is an alternation of hosts, various insects playing the part of definitive host. At least three species are pathogenic for man. These are pathogenic to a variable degree for some of the lower mammals, which in the wild state serve as "reservoirs" from which man may become infected through the agency of the insect host.

Trypanosoma gambiense is the parasite of African "sleeping sickness." Its detection in the blood is described on page 308. It is more abundant in the juice obtained by aspirating a lymph-gland with a large hypodermic needle, and in the late stages is also found in the cerebrospinal fluid. Its length varies from 15 to 33 μ, there being short stumpy forms, long slender forms, and intermediates. It is transmitted by a biting fly, *Glossina palpalis*. A second species which causes sleeping sickness in Africa has been named *T. rhodiense*. The chief point of distinction from *T. gambiense* is the situation of the nucleus close to, or even posterior to, the blepharoplast. It is transmitted by the fly, *Glossina morsitans*.

The antelope and other large game animals are probably the reservoir for these African trypanosomes.

Trypanosoma (*Schizotrypanum*) *cruzi* is the cause of Brazilian trypanosomiasis, or Chagas' disease, and in the febrile stage is found in the peripheral blood without much difficulty. Its average length is about 20 μ. The life cycle is very complicated. In the vertebrate host multiplication takes place in the muscles and certain internal organs where the parasites assume forms resembling Leishman-Donovan bodies. The early part of the flagellated stage is passed within red blood-corpuscles, the latter part free in the blood-plasma. The armadillo is probably the natural reservoir. The insect host, by which the trypanosome is transmitted to man,

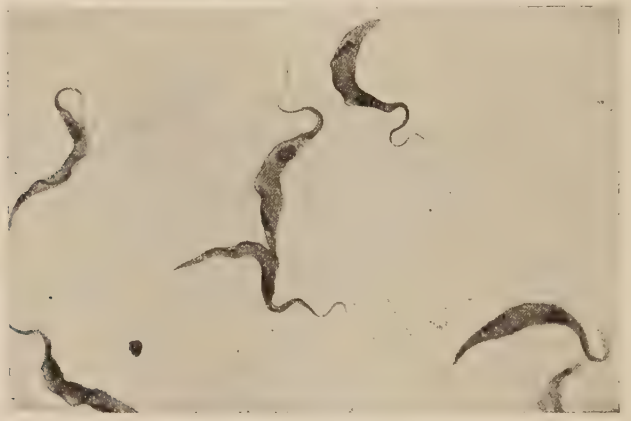


FIG. 191.—*Trypanosoma lewisi* in blood of rat. The red corpuscles were decolorized with acetic acid (photograph, × 1000).

is a large bug belonging to the genus *Triatoma* which is abundant in the dwellings of the poorer classes in Brazil. There are several species, of which *Triatoma megista* is most important and best known.

Trypanosoma lewisi, a very common and apparently harmless parasite of gray rats, especially sewer rats, is interesting because it closely resembles the pathogenic forms, and is easily obtained for study. Its posterior end is more pointed than that of *T. gambiense* (Fig. 191).

Trypanosoma evansi, *T. brucei*, and *T. equiperdum* produce respectively surra, nagana, and dourine, which are common and important diseases of horses, mules, and cattle in the Philippines, East India, and Africa.

6. Genus Leishmania.—The several species which compose this genus are apparently closely related to the trypanosomes, but their exact classification is undetermined. They have been grown outside the body and their transformation in cultures into flagellated trypanosome-like structures has been demonstrated.

They grow rather easily at room temperature either in citrated blood, or in N. N. N. (Novy, McNeal, Nicolle) medium (p. 646). Two to three weeks' incubation is necessary before calling cultures negative.

(1) **Leishmania donovani** is the cause of kala-azar, an important and common disease of India. With Wright's stain the

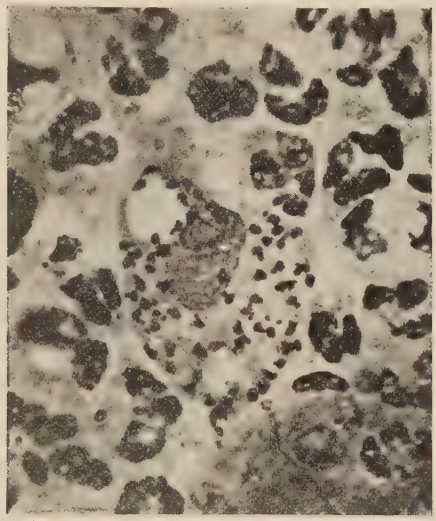


Fig. 192.—Smear from splenic pulp showing in the center a large mononuclear cell the cytoplasm of which is filled with Leishman-Donovan bodies. (Courtesy of C. I. Junkin.)

"Leishman-Donovan bodies" are round or oval, light blue structures, 2 to 3 μ in diameter, with two distinct reddish-purple chromatin masses, one large and pale (trophonucleus), the other small and deeply staining (blepharoplast) (Fig. 192). The parasites, which lie chiefly within endothelial cells, are especially abundant in the spleen; and splenic puncture has been resorted to for diagnosis, but is not without danger. They may also be found, although with less certainty, in material obtained by puncture of superficial lymph-glands. While they have been seen within endothelial leukocytes in the peripheral blood, particularly late in the disease,

they are extremely difficult to find in ordinary blood-films. The search may be greatly facilitated by concentrating the leukocytes. This can be done by the Bass and John method for malaria parasites (p. 317), although it is not necessary to carry the concentration beyond the second centrifugation. The leukocytes will form a whitish layer on top of the solidly packed red corpuscles. They are skimmed off with a capillary pipet, spread on a slide, and stained with Wright's stain. The bed-bug is probably the transmitting agent.

(2) **Leishmania infantum** is found in a form of splenomegaly with severe anemia occurring in young children in the region around the Mediterranean, especially Italy, and known as infantile kala-

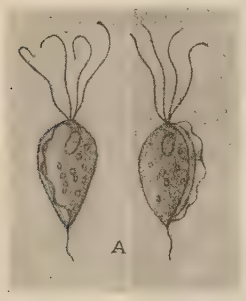


FIG. 193.—*Trichomonas hominis*, an intestinal flagellate (× 1000): Note the four anterior flagella and undulating membrane (composite drawing from various authors).

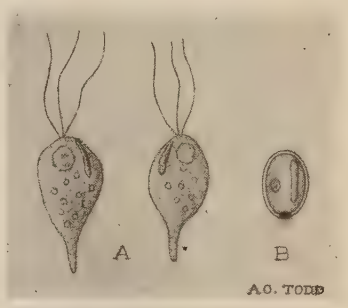


FIG. 194.—*Chilomastix mesnili*, an intestinal flagellate (× 1000): A, Flagellate form. Note the three anterior flagella, and the elongated cytostome or "mouth" (after Brumpt). B, Cyst. The cytostome can still be seen (Chandler, after Wenyon).

azar. Morphologically the parasite is indistinguishable from *L. donovani*. Dogs are the natural reservoir, and the dog-flea has been thought to transmit the disease, although recent work of Nicolle and Anderson would indicate that the flea is not implicated.

(3) **Leishmania tropica** resembles the preceding. It is found, lying intracellularly, in the granulation tissue of Delhi boil or Oriental sore. A variety, sometimes described as a separate species, *L. braziliensis* or *L. americana*, is similarly found in the ulcers of espundia, a very chronic form of mucocutaneous leishmaniasis, in South and Central America.

7. Genus Trichomonas.—(1) **Trichomonas hominis** is an oval or pear-shaped cell of somewhat changing shape (Fig. 193). The average length is about 10 to 15 μ, although there is consider-

able variation among individuals. In the anterior half of the cell is an oval nucleus which is not, however, well seen in unstained specimens; the cytoplasm contains food vacuoles. At the anterior or blunt end there is a cluster of four flagella of equal length, and along one side is an undulating membrane the thickened free edge of which is continued backward as a short flagellum. As to the number of flagella there is some confusion in the literature. Four is now accepted as the standard number, although there may be variation among individual parasites. Forms with three and five flagella have been called *Tritrichomonas* and *Pentatrichomonas*, respectively, but they are not generally regarded as separate species. Owing to the active motion of the flagella and undulating membrane these are not easily seen, and at first sight the parasite has much the appearance of a pus-corpuscle moving busily about among the fecal particles. A drop of Gram's iodin solution added to the preparation on the slide kills the parasites and brings out the internal structure and the flagella more clearly. In infested feces a few hours old, a characteristic degenerating form of *Trichomonas* is common. The organism exhibits peculiar undulating, almost ameboid movements which may continue for many hours. The usual habitat is the colon, but it is not infrequently found in other situations: in the vagina, in the mouth, especially about the gums, and in the sputum of persons suffering from pulmonary gangrene and putrid bronchitis.

Unlike the other intestinal flagellates, it does not, as far as is known, become encysted, although cysts have been most carefully sought, and supposed cysts have from time to time been described. Infection, therefore, probably takes place through ingestion of the flagellate forms, which, according to Hegner and Becker, appear to be sufficiently resistant to pass through the stomach without injury when swallowed with contaminated food.

Trichomonas hominis is common in the tropics, and from clinical reports appears to be wide-spread throughout the United States. It is probable, however, that in many cases the organisms reported were really *Chilomastix* or *Giardia*, which are more frequent. Surveys among soldiers from all parts of the country indicate its presence in about 3 per cent. of the population. Most authorities regard it as non-pathogenic; others believe that it may cause mild diarrhea of the dysenteric type, or that it at least may aggravate an

already existing inflammatory condition. The parasites are often so abundant that four or more may be seen in a single field of the high dry objective.

Special methods for finding this and other flagellates in feces are given in Chapter V.

8. Genus Chilomastix.—Chilomastix mesnili is widely distributed on all continents. Surveys among soldiers and others indicate that it is present in the feces of about 5 per cent. of all persons in this country, and is thus the second most prevalent intestinal flagellate, standing next below *Giardia*. Its pathogenicity is in dispute. It is a pear-shaped organism, 13 to 24 μ long, with three anterior flagella and no undulating membrane (Fig. 194). There is a large round nucleus not well seen in unstained specimens; and anteriorly is a large, elongated, heavily outlined cytostome or

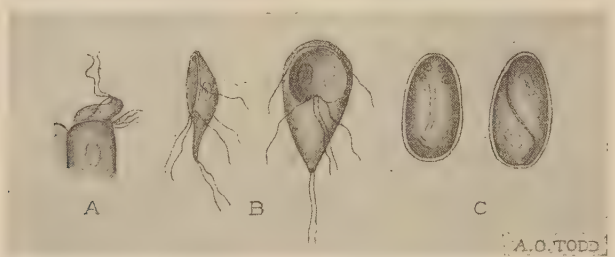


FIG. 195.—*Giardia lamblia*, the most prevalent intestinal flagellate: A, Flagellate form attached to the top of intestinal epithelial cell (× 500); B, flagellate form, side and ventral views (× 1000); C, cysts, frequently seen in the feces (× 1000). (After Chandler: A following Grassi and Schewiakoff, B and C following Wenyon.)

"mouth," within which is a small, slender flagellum not easily seen. The cytoplasm contains numerous small food vacuoles. The posterior extremity is projected into a narrow tail-like process. The usual habitat of *Chilomastix* is the large intestine.

The cyst is oval or, more often, pear shaped, and measures about 7.5 to 8.5 μ in length. The nucleus and the margins of the cytostome can be seen within it.

9. Genus Giardia.—Giardia lamblia (Lamblia intestinalis) is very common in the tropics and is probably the most prevalent of intestinal flagellates in this country. In a series of examinations of 2876 soldiers, as compiled by Kofoid, it was found 168 times. Other surveys place the incidence of infection among the general population as high as 12 per cent., while Maxcy found the incidence to be as high as 20 per cent. in a group of children examined

in Baltimore. However, Lynch found only 2.3 per cent. in 1040 cases in four years' experience. The parasite is generally considered of little pathogenic importance, but appears capable of causing a chronic enterocolitis with mild diarrhea which is very resistant to treatment. Closely related species are frequent in the intestines of rats, mice, rabbits, and guinea-pigs.

Giardia lamblia is pear shaped, measures 12 to 20 μ in length, and has a depression on one side of the blunt end by which it attaches itself to the tops of the epithelial cells of the intestinal wall. Three pairs of flagella are arranged about the depression and one pair at the pointed end (Figs. 195 and 196). Two nuclei can be

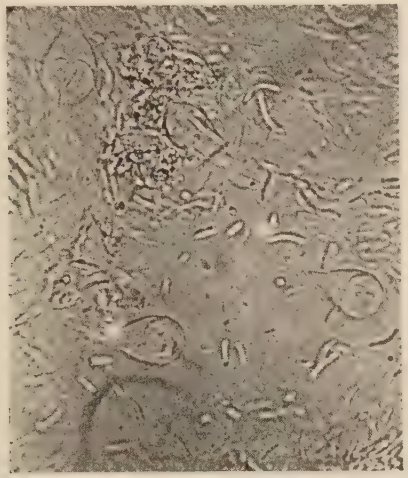


FIG. 196.—Vegetative form of *Giardia lamblia* in feces; photomicrograph (about × 800.)

made out. The cytoplasm does not contain food vacuoles. Its usual habitat is the upper part of the small intestine, especially the duodenum.

Giardia is disseminated in the encysted stage, and unless active diarrhea from the small intestine exists, or the stool is obtained by catharsis, only encysted forms are likely to be found. These may be extremely numerous and, while less conspicuous than the motile individuals, should not be difficult to find and identify even by one who is not familiar with protozoal cysts. The cysts are oval, measure about 8 × 14 μ, have the internal structure shown in Fig. 195, and are surrounded by a fairly thick hyaline wall. Boeck found

their longevity outside the body to be thirty-four days in water and their thermal death-point 64° C.

Other Intestinal Flagellates.—The literature relating to the intestinal flagellates and their pathogenicity is much confused. Probably the only important members of the group in this country are the three described above. Other species which are very rarely found may be mentioned briefly. *Embadomonas intestinalis* has two anterior flagella and a definitely outlined cytostome, and its cyst resembles that of *Chilomastix*, but is only about 5 μ long. *Enteromonas hominis* usually has three anterior flagella, and a fourth which arises anteriorly, trails backward, and is adherent to the body. These two flagellates are small, 4 to 8 μ in length. *Tricercomonas intestinalis*, formerly described, is now, by good authorities, identified with *Enteromonas*. *Cercomonas*, with two anterior flagella, one free, one directed backward, attached to the body, and projecting posteriorly, is, contrary to the older belief which was due to confusion with *Chilomastix* and *Trichomonas*, very rare in fresh feces. *Bodo*, with a single nucleus and two anterior flagella, is also very rare. *Cercomonas* and *Bodo* probably do not live within the human body, but are to be classed as "coprozoic organisms" which reach the feces as contaminations and there find a favorable medium for growth.

Special methods of examining feces for flagellates and other protozoa are given on pages 438 and 439.

CLASS SPOROZOA

Subclass Telosporidia

All the members of this class are parasitic, but only a few have been observed in man, and only one genus, *Plasmodium*, is of much importance in human pathology. Propagation is by means of spores, and sporulation ends the life of the individual. In some species there is an alternation of generations, in one of which sexual processes appear. In such cases the male individual may be provided with flagella. Otherwise there are no special organs of locomotion.

1. Genus Eimeria.—Eimeria stiedæ (Coccidium cuniculi).—This is a very common parasite of the rabbit, and has been much studied, but extremely few, if any, authentic cases of infection in man have been reported. The parasite, which when fully developed is ovoid in shape and measures about 30 to 50 μ in length and has a shell-like integument, develops within the epithelial cells of the

bile-passages. Its presence causes the formation of whitish nodules, usually with caseous contents. On reaching adult size it divides into a number of spores or merozoites, which enter other epithelial cells and repeat the cycle. A sexual cycle which suggests that of the malarial parasite, but does not require an insect host, also occurs. By conjugation of microgamete with macrogamete a zygote is formed. This develops a definite membraneous wall and thus becomes an *oöcyst* (Fig. 197) which passes out with the feces. Its contents then divide into a number of sporozoïtes. The cyst remains quiescent until it reaches the stomach of a new host, usually through contaminated food. Here the cyst wall is digested and the sporozoïtes are set free to travel to the liver and enter epithelial cells, where they initiate a new cycle.

A number of related species have in very rare instances been found in man. In most of these the oöcyst was large and spherical.

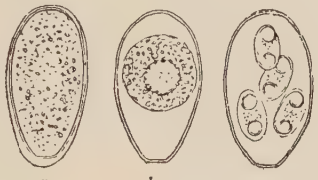


FIG. 197.—*Eimeria stiedæ*, from the liver of the rabbit, oöcysts in various stages of development (after Leuckart).

2. Genus Isospora.—Isospora hominis.—This is closely related to the preceding and has a similar life-history. It is a parasite of man, its habitat being the epithelial cells of the villi of the small intestine. Apparently it causes no particular disturbance. Diagnosis depends upon the recognition of oöcysts in the feces. These are colorless, ovoid bodies, measuring about 14 by 28 μ, with a clear-cut definite wall usually with two or more layers. When they first pass out of the body the protoplasm is unsegmented and appears as a rounded granular mass which does not fill the cyst, as in Figure 197, *b*. They might easily be mistaken for the egg of some unknown worm. To gain an idea of their general appearance one may study the contents of the whitish nodules in the liver of a rabbit infested with the coccidium described above.

Isospora hominis has been reported chiefly in British soldiers coming from Gallipoli, Salonika, and the Balkans. A case in which

the disease may possibly have been acquired in the United States has been reported by Haughwout.

3. Genus Plasmodium.—This genus includes the malarial parasites which have already been fully described (pp. 308–319).

4. Genus Babesia.—The proper position of this genus is uncertain. It is placed among the flagellates by some. The chief member is *Babesia* (*Piroplasma*) *bigeminum*, the cause of Texas fever in cattle. It is a minute, pear-shaped organism, lying in pairs within the red blood-corpuscles. An organism described as occurring in the red blood-corpuscles in Rocky Mountain spotted fever was at one time placed in this genus under the name *B.* (*Piroplasma*) *hominis*. While the work of Wolbach[1] and others suggests the protozoal origin of the disease, the classification of the parasite remains entirely unsettled. Wolbach has named it *Dermacentroxenus rickettsi*. It and similar very minute lancet-shaped structures found in typhus and trench fevers are included under the name "rickettsia bodies." Wolbach found it in large numbers in the endothelial and smooth muscle-cells of the smaller blood-vessels, but never in direct blood-smears. It offers no help in diagnosis. The disease is transmitted by ticks, chiefly *Dermacentor andersoni* (Fig. 244).

CLASS INFUSORIA

Subclass Ciliata

The conspicuous feature of this class is the presence of cilia. These are hair-like appendages, which have a regular to-and-fro motion instead of the irregular lashing motion of flagella. They are also shorter and more numerous than flagella, and usually cover the greater part of the surface. Most infusoria are of fixed shape and contain two nuclei. Contractile and food vacuoles are also present. Encystment is common. Only one species is of medical interest. Certain ciliated structures, which have been described as infusoria, notably in sputum and nasal mucus, were probably ciliated body cells.

1. Genus Balantidium.—(1) **Balantidium coli.**—This parasite, formerly called *Paramœcium coli*, is an occasional inhabitant of the colon of man, where it penetrates into the mucous membrane and produces a diarrheal condition resembling amebic dysentery.

[1] Wolbach, S. B.: Studies on Rocky Mountain Fever, Jour. Med. Research, vol. 41, p. 1, November, 1919.

Infection is most frequent among farmers, and in some cases has been associated with the symptoms of pernicious anemia. It is an actively moving oval organism, about 60 to 100 μ long and 50 to 70 μ wide, is covered with cilia, which are arranged in longitudinal rows, giving a striated appearance, and contains a bean-shaped macronucleus, a globular micronucleus, two contractile vacuoles, and variously sized granules (Figs. 198 and 199). At the anterior end is a funnel-shaped mouth. The parasite is so large that it can hardly be overlooked if present upon the slide and still active.

Its ordinary habitat is the large intestine of the domestic pig, where it apparently causes no disturbance. It probably reaches man in the encysted condition.

FIG. 198.—*Balantidium coli* (about × 350) (after Eichhorst).

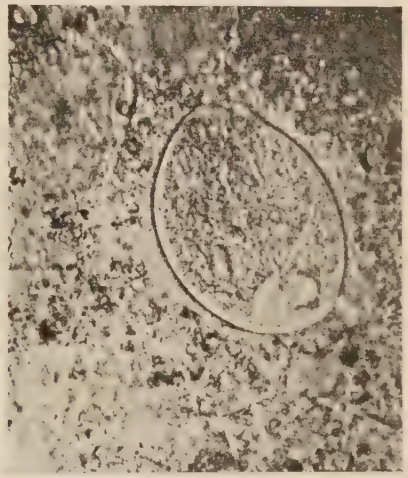


FIG. 199.—Photomicrograph of *Balantidium coli* in feces (about × 500).

(2) **Balantidium minutum** resembles *B. coli*, but is smaller, measuring 20 to 30 by 15 to 20 μ. It has been found a few times in diarrheal stools.

PHYLUM PLATYHELMINTHES

The old phylum Vermidea has been subdivided into three phyla, those which are of interest here being the Platyhelminthes and Nemathelminthes, the flat worms and the round worms respectively. Of these, many species are parasitic in man and the higher animals. In some cases man is the regular host; in others the usual habitat is some one of the animals, and the occurrence of the worm in man is more or less accidental. Such are called *incidental parasites*. Only those worms that are found in man

with sufficient frequency to be of medical interest are mentioned here.

The most important means of clinical diagnosis of infection by either the flat worms or the round worms is the finding of ova. In many cases the ova are so characteristic that the finding of a single one will establish the diagnosis. In other cases they must be carefully studied and a considerable number measured. While ova from the same species will naturally vary somewhat, the average size of a dozen or more is pretty constant, probably as much so as is the case with different species of birds. The measurements given here are mainly those accepted by Stiles or Ward.

PHYLUM PLATYHELMINTHES

(Flat Worms)

CLASS **Trematoda.**—Flukes. Unsegmented, leaf-shaped, with alimentary tract.

Genus	*Species*
Fasciola.	*F. hepatica.*
Opisthorchis.	*Op. felineus.*
Clonorchis.	*C. sinensis.*
Fasciolopsis.	*F. buski.*
Paragonimus.	*P. westermanii.*
Schistosoma.	*S. hæmatobium.*
	S. mansoni.
	S. japonicum.

CLASS **Cestoda.**—Tapeworms. Segmented, ribbon-shaped, with no alimentary tract.

Genus	*Species*
Tænia.	*T. saginata.*
	T. solium.
	T. echinococcus.
Hymenolepis.	*H. nana.*
	H. diminuta.
Dipylidium.	*D. caninum.*
Diphyllobothrium.	*D. latum.*

CLASS TREMATODA

The trematodes, commonly known as "flukes," are flat, unsegmented, generally tongue- or leaf-shaped worms. They are comparatively small, most species averaging between 5 and 15 mm. in length. Trematode infection is uncommon in this country and nearly all the cases are imported.

Most species have two radially striated sucking disks: an oral sucker surrounding the mouth at the anterior end, and a ventral sucker on the ventral surface of the anterior third of the body. The digestive tract is incomplete, without anus. The short esophagus divides into two intestines which pass backward in the lateral zones of the body and end as blind tubes. In some cases, for example, *F. hepatica*, the intestine gives off many lateral branches.

The nervous system consists merely of two ganglia with connecting fibers and several fine nerves which run posteriorly.

The excretory system includes numerous scattered excretory cells and a series of fine canals which unite to form an excretory duct which opens to the surface in an excretory pore situated dorsally or posteriorly.

Respiratory and circulatory systems are lacking.

Nearly all species are hermaphroditic, having the reproductive organs of both sexes. The male organs consist of two or more testes, usually situated near the center or back part of the body, and a spermatic duct which ends in a small cirrus or copulatory organ at the genital pore. This genital opening is generally situated near the ventral sucker. The female organs consist of an ovary, usually lying in front of the testes, a short oviduct, and a coiled tubular uterus, which opens to the surface at the genital pore. The oviduct receives the secretion from the numerous vitelline or yolk glands which lie along the lateral borders of the parasite. The junction of the oviduct and uterus is known as the oötype and receives the shell materials from neighboring shell glands. Most of the above-mentioned structures are shown in Figures 200 and 202.

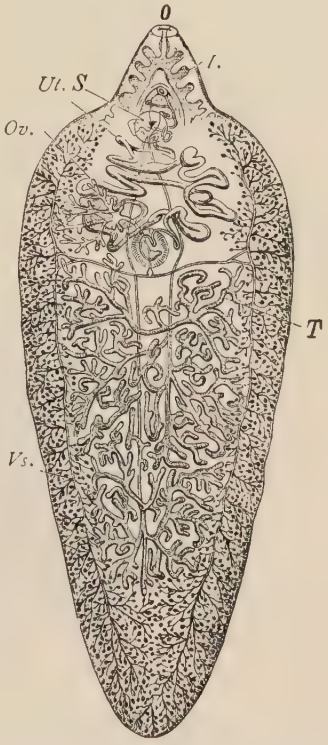


FIG. 200.—The liver-fluke *Fasciola hepatica*, showing internal structure: *I.*, Intestine; *Vs.*, vitelline glands; *Ov.*, ovary; *O*, oral aperture; *Ut.*, uterus; *S*, ventral sucker; *T*, testes. The two vasa efferentia can be seen running upward in the midline. The branches of the intestine are shown only in the head-cone. Intermediate host is a water snail, *Limnæus trunculatus* Müll. (After Claus.)

The germinal cells are fertilized in the oviduct by spermatozoa derived from the same or another individual. These enter by way of the

genital pore and uterine canal. After fertilization the yolk material is added, and the egg then receives the shell substance and passes on into the uterus and thence to the genital pore. The diagnosis of fluke infection rests upon the finding of eggs. The number of eggs is enormous, sometimes running into the tens of thousands. The eggs of nearly all species are operculated (provided with a lid), the only important exception being the several species of the genus *Schistosoma*. This genus also differs from the other flukes in that the sexes are separate.

Development takes place by a complicated process of alternation of generations, the intermediate host being usually a snail, mussel, or crab. In some cases there are two intermediate hosts, parasitized in succession. The life-history of *Fasciola hepatica*, given on page 477, may be taken as typical of the group.

FIG. 201.—Egg of the liver-fluke, *Fasciola hepatica*, with lid forced open by pressure on the cover-glass (photograph, × 250).

1. Genus Fasciola.—Fasciola hepatica.—The "liver fluke" inhabits the bile-ducts of numerous herbivorous animals, especially sheep, where it is an important cause of disease. It brings about obstruction of the bile-passages, with enlargement and degeneration of the liver—"liver rot." This fluke is readily obtained for study. The adult fluke is leaf shaped, the average size being about 2.8 by 1.2 cm. The anterior end projects like a beak (head-cone 3 to 4 mm. long—Fig. 200). The numerous small vitelline glands lie along the lateral and posterior borders, giving a granular appearance which in stained specimens sharply marks off this zone from the central field of the body. The central field, which has much the shape of the parasite itself, is occupied in its posterior two-thirds by the branching testicular tubules and anteriorly by the ovary and coiled tubular uterus which is usually easily seen because of the eggs which

it contains. Ova appear in the feces of the host. They are yellowish brown, oval, operculated, and measure about 130 to 140 by 75 to 90 μ.

The life-history of *F. hepatica* is given in some detail because it is typical of the group. The eggs pass out with the feces of the host. After two or three weeks the embryo develops into a ciliated body resembling a ciliated infusorian, and called a *miracidium*. If the eggs reach water the miracidium escapes from the shell and swims about until it finds a certain water snail, *Limnæus trunculatus* Müll, into which it penetrates and lodges in the pulmonary cavity. Here it changes into a cyst-like structure, from the inner or germinal layer of which there develop a number of small worm-like organisms called *rediæ*. These migrate to other parts of the snail's body and produce a second type of larvæ called *cercariæ*, which have much the form of minute tadpoles. Under certain conditions the rediæ produce a second generation of rediæ which, in turn, produce the cercariæ. The cercariæ leave the host, swim about in the water, and finally attach themselves to submerged blades of grass, lose their tails, and become encysted. There they remain until taken into the digestive tract of a sheep or other appropriate host. Here they again become free and find their way up the bile-passages to the liver, where they develop into adult flukes. In the case of certain other flukes (for example, the lung fluke) the cercariæ do not encyst upon grass-blades, but enter the bodies of certain aquatic animals (fish, crabs) and there encyst.

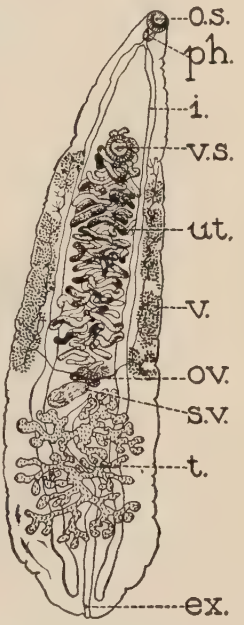


Fig. 202.—*Clonorchis sinensis*, showing internal structure: *o.s.*, Oral sucker; *ph.*, pharynx; *i.*, intestine; *v.s.*, ventral sucker; *ut.*, uterus; *v.*, vitellaria; *ov.*, ovary; *s.v.*, vesicula seminalis; *t.*, testis; *ex.*, excretory duct. *Bythinia striatula* var. *japonica* is first intermediate host (× 6). (From Abt's Pediatrics.)

2. Genus Opisthorchis.—Opisthorchis felineus inhabits the gall-bladder and bile-ducts of the domestic cat and a few other animals. Infection in man has been repeatedly observed in Europe, and especially in Siberia. The body is flat, yellowish-red in color,

and almost transparent. It measures 8 to 11 by 1.5 to 2 mm. The eggs, which are found in the feces, are oval, with a well-defined operculum at the narrower end, and contain a ciliated embryo when deposited. They measure about 30 by 11 μ. Infection takes place through eating of insufficiently cooked fish. The life-history is unknown, but the last intermediate host is a fish (either *Idus melanotus*, *Leuciscus rutilus*, or *Tinca tinca*)

3. Genus Clonorchis.—Clonorchis sinensis (Fig. 202), like the preceding fluke, inhabits the gall-bladder and bile-ducts of domestic cats and dogs. It is, however, much more frequent in man, being a common and important parasite in certain parts of Japan and China. The number present may be very great; over 4000 were counted in one case. The parasite resembles *Opisthorchis felineus*

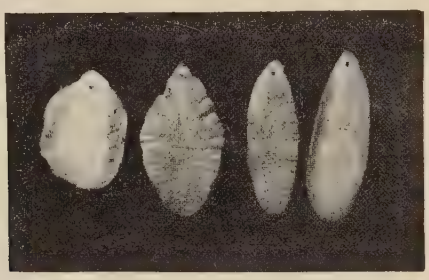


FIG. 203.—Photographs of *Fasciolopsis buski*, showing the appearance of fresh specimens (F. W. Goddard in The Jour. of Parasitology, June, 1919). Intermediate hosts, unknown species of either *Planorbis* or *Segmentina*, fresh-water snails.

in shape and color. It is 10 to 14 mm. long and 2.5 to 4 mm. broad. The eggs have a sharply defined lid and measure 25 to 30 by 15 to 17 μ. When they appear in the feces they contain a ciliated embryo. The first larval stage is passed in a species of mollusk, *Bythinia striatula*, var. *japonica;* the second, in several species of fish, from which the larva passes to man when the fish are eaten without sufficient cooking.

4. Genus Fasciolopsis.—Fasciolopsis buski.—This fluke (Fig. 203) is parasitic in the duodenum of man, and is wide-spread in the East, notably in India, China, and Japan. A few imported cases have been reported in this country. When in considerable numbers it causes a bloody diarrhea accompanied by fever. It is the largest of the flukes. The usual length is about 30 mm.; width, 10 to 12 mm.; thickness, 2 to 3 mm. The eggs are thin shelled, with

granular contents, possess a minute operculum, and measure about 135 by 80 to 85 μ

Barlow has worked out completely the life-history of this fluke. Ninety-one days or more are required for the development of the adult fluke from the ovum. The cercariæ after leaving the intermediate host encyst on water plants that are eaten raw by the Chinese. Carbon tetrachlorid proved an effective anthelmintic in Barlow's experience.

5. Genus Paragonimus.—Paragonimus westermanii, called the "lung fluke," is also a common parasite of man in Japan, China, and Korea. It inhabits the lung, causing the formation of

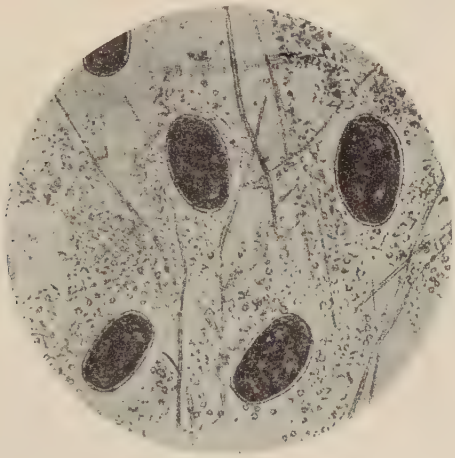


FIG. 204.—Sputum of man containing eggs of the lung fluke, greatly enlarged (after Manson).

small cavities. Moderate hemoptysis is the principal symptom. Ova are readily found in the sputum (Fig. 204); the worms themselves are seldom seen, except *postmortem*. The worms somewhat resemble a coffee-bean in size and shape. They are faintly reddish-brown in color, egg shaped, with the ventral surface flattened, and measure 8 to 10 by 4 to 6 mm. The ova are thin shelled, operculated, brownish yellow, and measure from 87 to 100 by 52 to 66 μ.

There are two intermediate hosts, a mollusk in which the cercariæ are formed, and a fresh-water crab (a common article of food in Japan) in which they encyst. The encysted forms have also been found in fresh-water snails.

According to Ward, three distinct species have been confused

under the name *P. westermanii:* the original form, *P. westermanii,* found in the tiger; the American lung fluke, *P. kellicotti,* thus far found only in cat, dog, and hog; and the Asiatic lung fluke of man, *P. ringeri,* described above.

6. Genus Schistosoma.—(1) **Schistosoma hæmatobium.**—This trematode, frequently called *Bilharzia hæmatobia,* is an extremely common cause of disease (bilharziasis or Egyptian hematuria) in Northern Africa, particularly in Egypt.

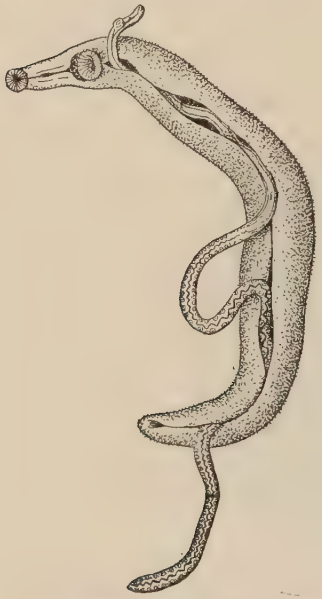


Fig. 205.—A blood-fluke, *Schistosoma hæmatobium,* male and female (× 12) (after Looss). Intermediate hosts are snails, *Bullinus brocchi, Physopsis africana.*

Unlike the other flukes, the sexes are separate. The male is 12 to 14 mm. long and 1 mm. broad. The body is flattened and the lateral edges curl ventrally, forming a longitudinal groove, in which the female lies (Fig. 205). The latter is cylindric in shape, about 20 mm. long and 0.25 mm. in diameter. The eggs are an elongated oval, about 120 to 190 μ long, and 50 to 73 μ broad, yellowish in color, and slightly transparent. They possess no lid such as characterizes the eggs of most of the trematodes, but are provided with a thorn-like spine which is placed at one end (Fig. 206). Within is a ciliated embryo.

In man the worm lives in the veins, particularly those of the bladder and rectum, leading to obstruction and inflammation. The eggs penetrate into the tissues and are present in abundance in the mucosa of the bladder and rectum. They also appear in the urine and, less commonly, in the feces.

The life-history is similar to that given for *Fasciola hepatica.* A species of snail serves as intermediate host, and infestation of man apparently takes place both by mouth and through the skin. The tadpole-like cercariæ have forked tails.

(2) **Schistosoma mansoni.**—It has long been observed that schistosoma eggs in the urine have usually a terminal spine, while in the feces the lateral spine is more common. It is now known

that the lateral-spined egg is that of a distinct species, to which the name *Schistosoma mansoni* has been given. It is found in

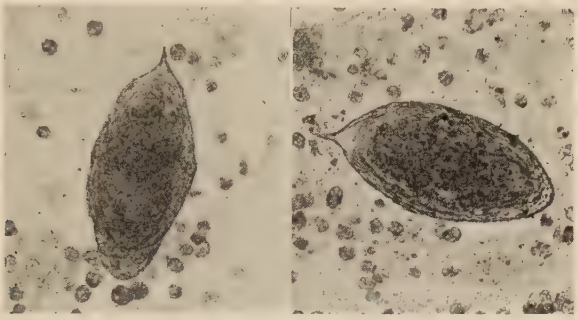


FIG. 206.—Ova of *Schistosoma hæmatobium* with pus-corpuscles in urine (photographs, × 250).

Africa along with *Schistosoma hæmatobium*, but is especially prevalent in the West Indies and Central America. The adult worms

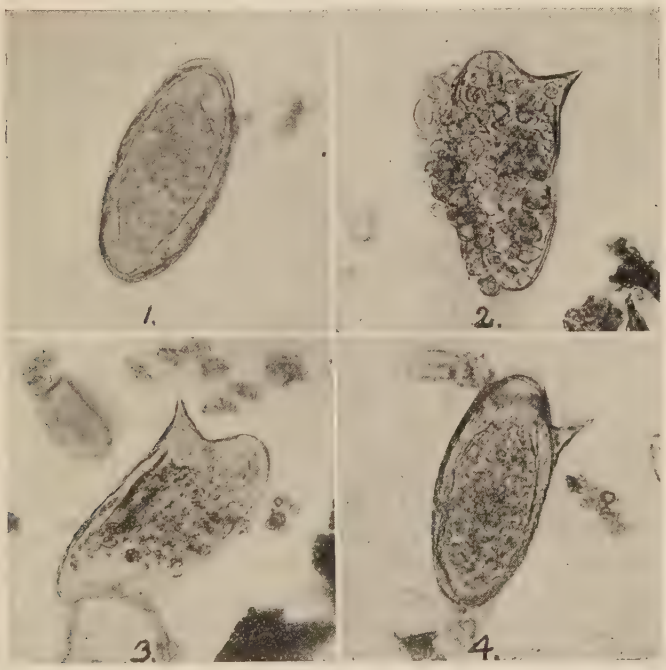


FIG. 207.—Ova of *Schistosoma mansoni:* 1, With spine out of focus; 2, in a clump of red blood-cells; 3, apparently unfertilized; 4, usual appearance (photographs, × 250). Intermediate hosts are snails, *Planorbis boissyi*, or *Planorbis centrimentralis*.

closely resemble the male and female of *S. hæmatobium*. They inhabit the rectal and portal veins, and ova appear in the feces, where

they are very easily recognized from their size and the characteristic spine (Fig. 207 and Plate XI). They are light yellow in color, measure 112 to 162 by 60 to 70 μ, and are provided with a clean-cut, sharply-pointed spine, which is situated at the juncture of the last and third quarters of the egg, and is directed backward. Within the egg is a ciliated embryo (miracidium) which can be seen without difficulty. The life-history is very similar to that of *S. hæmatobium.*

(3) **Schistosoma japonicum** resembles *S. hæmatobium* morphologically, but both the male and female are smaller. The ova, which appear in the feces, are ovoid, thin shelled, and without lid or spine. They average 83 by 62 μ in size, and contain a ciliated embryo. The worm inhabits the portal, and probably also other veins. A fresh-water snail (some species of *Planorbis*) serves as intermediate host. The rice fields are often the place of infection.

Class Cestoda

The cestodes, or tapeworms, are very common parasites of both man and the animals. In the adult stage they consist of a linear series of flat, usually rectangular segments (proglottides), at one end of which is a smaller segment, the scolex or "head and neck," especially adapted by means of sucking disks and hooklets for attachment to the host. The series represents a colony, of which the scolex is ancestor. The proglottides are sexually complete hermaphroditic individuals which are derived from the scolex by budding, and the segment most distant from the scolex is thus the oldest. With the exception of the immature segments near the scolex each contains a uterus filled with ova.

In general each proglottis contains the same reproductive organs with the same functions as have been described for the trematodes (p. 475). These are not found in the young segments adjacent to the scolex, but are fully developed in the middle and latter part of the chain, the male organs maturing first. The testes generally consist of a great number of very small glands scattered throughout the segment. Their ducts unite to form a single vas deferens leading to the genital pore, which is situated either upon one lateral border of the segment or upon its flat surface.

The sperms from the same or another proglottis reach the genital pore and are carried by a tubular vagina to the oviduct which is situated in the posterior part of the proglottis. Here the germinal cells derived from the two ovaries are fertilized and receive their yolk material which

is the secretion of numerous vitelline glands as in the case of the trematodes. The eggs pass into the oötype, where the shell substance is added, and thence on into the uterus. The uterus is either a convoluted tube which opens in a birth pore (separate from the genital pore) upon a flat surface of the segment, usually anteriorly; or it is a closed sac which extends longitudinally in the midline, and as it fills with eggs sometimes comes to have lateral branches. In the latter case there is no birth pore and the eggs accumulate until the segment is packed with them, when the male organs and sometimes also the ovaries atrophy; the eggs are liberated only when the proglottis disintegrates.

The excretory apparatus consists of numerous scattered excretory cells with capillaries which empty into four excretory canals running the

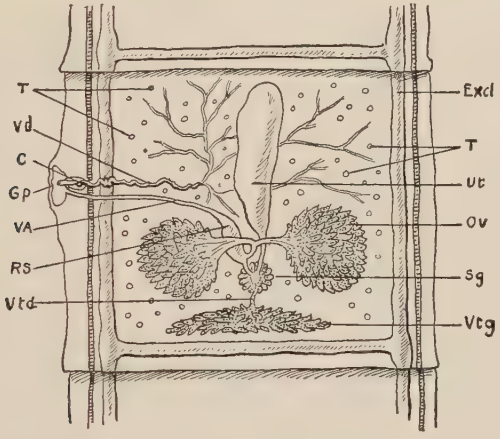


FIG. 208.—Diagram of the anatomy of a tapeworm (*Tænia saginata*): *T*, Testes; *Vd*, vas deferens; *C*, cirrus; *Gp*, genital pore; *Va*, vagina; *Rs*, receptaculum seminis; *Vtg*, vitelline glands; *Vtd*, vitelline duct; *Sg*, shell gland; *Ov*, ovaries; *Ut*, uterus, side branches not yet developed; at the sides are the two longitudinal nerves, also the excretory canals (*Excl*), which are connected by a transverse canal in each segment. (From Rivas' Human Parasitology.)

full length of the chain, two near each lateral border, and emptying posteriorly. In some species these communicate by a cross canal in each proglottis. Also running the length of the chain, near the lateral borders, are two or more nerve cords derived from ganglia in the scolex. There is no digestive system, the parasite taking its nourishment by absorption through its surface. Circulatory and respiratory systems are also absent.

All the tapeworms pass a larval stage in the tissues of an intermediate host, which is rarely of the same species as that which harbors the adult worm. Within the ova which have developed in the proglottides of the adult worm, and which pass out with the feces of the host, there develop

embryos, or *oncospheres*, each provided with three pairs of horny hooklets. When the egg is taken into the intestine of a suitable animal the oncosphere is liberated and penetrates to the muscles or viscera, and there, in the case of most of the tapeworms, forms a cyst in which develop usually one, but sometimes many, scolices, which are identical with the head of the adult worm. When the flesh containing this cystic stage is eaten without sufficient cooking to destroy the scolices the latter attach themselves to the intestinal wall and produce adult tapeworms by budding. The oncosphere of some of the tapeworms leaves the egg in the open and exists for a time as a free-living larva before entering the intermediate host.

Ordinarily only the adult stage occurs in man. In the case of *Tænia echinococcus* only the larval stage is found. *T. solium* may infest man in either stage, although the cystic stage is rare.

The large tapeworms, *Tænia saginata*, *T. solium*, and *Diphyllobothrium latum*, are distinguished from one another mainly by the structure of the scolex and of the uterus. The scolex should be studied with a low-power objective or a hand-lens. The uterus is best seen by pressing the segment out between two glass slides.

Usually the patient picks out the main body of the worm and brings it in triumph, but too often the small head is lacking and one is left to guess whether it was lost with the feces. After a considerable portion of the worm has been expelled, some weeks or months may elapse before ova or segments again appear in the feces.

Since the head, or scolex, is the ancestor from which the worm is formed in the intestine, it is important, after giving a vermifuge, to make certain that the head has been passed with the worm. Should it remain, a new worm will develop. The technic described by Magath and Brown has proved most satisfactory at the Mayo Clinic. The patient is prepared by having him abstain from lunch and supper on the day previous to treatment, although black coffee or tea may be taken freely. At six P. M. he takes from 15 to 30 gm. magnesium sulphate, and the following morning at six A. M. he takes the same dose. He is allowed no breakfast, and after his bowels have moved, he is given 30 c.c. of the following emulsion: Oleoresinæ aspidii 3 gm.; pulv. acaciæ 4 gm.; aqua dist. qs. ad. 30 c.c. One hour later he is given a second 30 c.c. of the same emulsion. Two hours later he is given 30 gm. of magnesium sulphate; two hours after this a large soap-suds enema is given. All

stools are saved in a container and taken, together with the stool passed before the administration of the drug, directly to the laboratory. To make sure of finding the head the entire quantity of feces is passed through a coarse sieve.

The principal tapeworms found in man belong to the genera Tænia, Hymenolepis, and Diphyllobothrium.

1. Genus Tænia.—(1) **Tænia saginata** (Fig. 209).—This, the beef tapeworm, is the common tapeworm of the United States, and is widely distributed over the world. Its length is generally about 4 to 8 meters. The scolex is about the size of a large pin-head

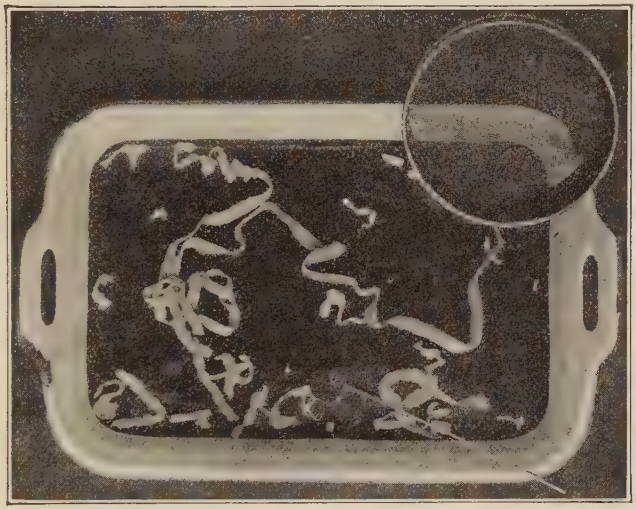


FIG. 209.—Apparatus used for recovery of the heads of tapeworms. The sieve is a No. 20 mesh. The enamel pan is painted black with asphalt paint. The wooden applicator points to the head which is relatively much smaller than the segments usually seen by the patient.

(1.5 to 2 mm. in diameter), and is surrounded by four sucking disks, but has no hooklets (Fig. 210). The neck is about 1 mm. wide. The terminal segments, which become detached and appear in the feces, measure about 18 to 20 mm. long by 4 to 7 mm. wide. The genital pore lies at the top of an elevation upon one of the margins of the segment, and the side upon which it is situated alternates in adjoining segments. The uterus extends along the midline of the segment and gives off twenty to thirty branches upon each side (Fig. 220, *a*). There is no birth pore. Malformations of the segments are frequent.

The larval stage is passed in the muscles of various animals, especially cattle. It rarely or never occurs in man, hence there is little or no danger of infection from examining feces.

FIG. 210.—Head of *Tænia saginata*, showing four sucking disks (× 15).

The scolex is ingested with the meat, its capsule is dissolved by the digestive juices, and it attaches itself to the intestinal wall by means of its suckers. It then develops into the mature worm, which may grow very rapidly, even as many as ten segments being formed in a day.

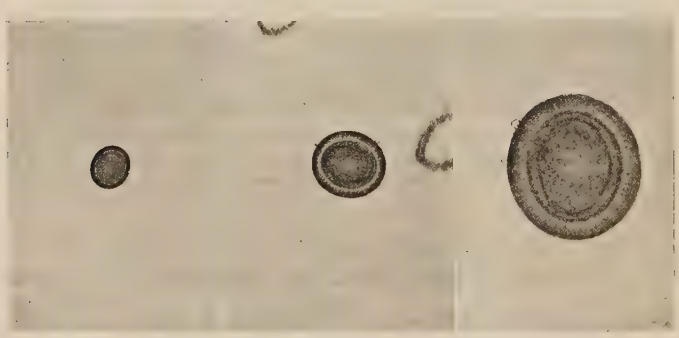


FIG. 211.—Eggs of *Tænia saginata*, magnifications 100, 250, and 500 diameters (photographs).

Diagnosis rests upon the finding of segments, which usually come away singly, or of ova, in the feces. Since there is no birth-pore the ova appear only when ripe segments disintegrate in the intestine, which, however, is a common occurrence. They are spheric or ovoid, yellow to brown in color, and have a thick, radially

striated cortex which is commonly called the shell, although strictly speaking it is not a true egg-shell since it is produced by the embryo (Fig. 211 and Plate XI). Within them the six hooklets of the embryo (oncosphere) can usually be made out as three pairs of parallel lines. The size of the ova varies from 20 to 30 μ wide and 30 to 40 μ long. Surrounding the egg, particularly when pressed out from the worm, is sometimes seen a broad transparent slightly granular zone called the vitelline membrane. Vegetable cells, which are generally present in the feces, are often mistaken for these ova, although there is seldom any great resemblance.

(2) **Tænia solium,** the pork tapeworm, is very rare in this country. It is usually much shorter than *Tænia saginata.* The scolex is about 0.6 to 1 mm. wide, is surrounded by four sucking disks, and has a projection, or rostellum, with a double row of horny hooklets, usually twenty-six to twenty-eight in number (Fig. 212). The terminal segments measure about 5 to 6 by 10 to 12 mm. The uterus has only seven to fourteen branches on each side (Fig. 220, *b*).

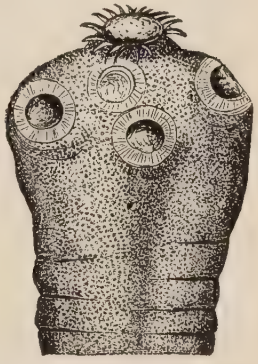


FIG. 212.—Head of *Tænia solium* (Mosler and Peiper).

The cysticercus stage occurs ordinarily in the muscles of the pig, but is occasionally seen in man, most frequently affecting the brain and eye (*Cysticercus cellulosæ*). There is, therefore, danger of infection from handling feces.

The ova so closely resemble those of *Tænia saginata* as to be practically indistinguishable. They average about 31 to 36 μ in diameter and are usually spheric (Plate XI).

(3) **Tænia echinococcus.**—The mature form of this tapeworm inhabits the intestines of the dog and wolf, never of man. The larvæ develop in cattle and sheep ordinarily, but are sometimes found in man, where they give rise to echinococcus or "hydatid" disease. The condition is unusual in North America, but is not infrequent in Central Europe, and is common in Greece, Iceland, Australia, and South America.

The adult parasite is 2.5 to 5 mm. long, and consists of only four segments (Fig. 213). It contains many ova, which resemble those of *Tænia saginata.* When the ova reach the digestive tract

of man the embryos are set free and find their way to the liver, lung, or other organ, where they develop into cysts, thus losing their identity. The cysts grow very slowly, and after many years may attain the size of a child's head. Other cysts, called "daughter-cysts," are formed within these. The cyst wall is made up of two layers, from the inner of which (the so-called "brood membrane") there develop larvæ which are identical with the head, or scolex, of the mature parasite. These are ovoid structures 0.2 to 0.3 mm. long. Each has four lateral suckers and a rostellum surmounted by a double circular row of horny hooklets. The rostellum with its hooklets is frequently invaginated into the body.

Diagnosis of echinococcus disease may be made in several ways:

FIG. 213.—*Tænia echinococcus*, enlarged (Mosler and Peiper).

(*a*) The complement-fixation method is reliable. The best antigen is cyst fluid, filtered through cotton and preserved with 0.5 per cent. phenol, and the dose used in the test is one-third or one-fourth the anticomplementary unit. It keeps well, but just before use it should be heated at 60° C. for from fifteen to thirty minutes to reduce anticomplementary activity. Fluid from cysts in sheep can probably be obtained from the abattoirs of the large packing houses by special arrangement. Adequate controls should be run, including a strongly positive syphilitic serum.

(*b*) The precipitin test is described on page 581.

(*c*) The cutaneous reaction, using cyst fluid applied upon the skin, as in von Pirquet's test, or intradermally, as in the luetin test, is also reliable if adequate control tests be made. The reaction may appear within a few minutes.

(*d*) Microscopic examination of the cyst fluid obtained at operation or autopsy is decisive. It is, however, unwise to make an exploratory puncture because of the danger of leakage of the fluid into the tissues or peritoneal cavity. This might induce anaphylactic shock or, at least, spread the disease by implantation. The cyst fluid is clear, between 1.009 and 1.015 in specific gravity, and contains a notable amount of sodium chlorid, but no albumin. Positive identification of the fluid depends upon detection of scolices, free hooklets which have fallen off from degenerated scolices,

or particles of cyst wall which are characteristically laminated, and usually have curled edges. The lamination is best seen at the torn

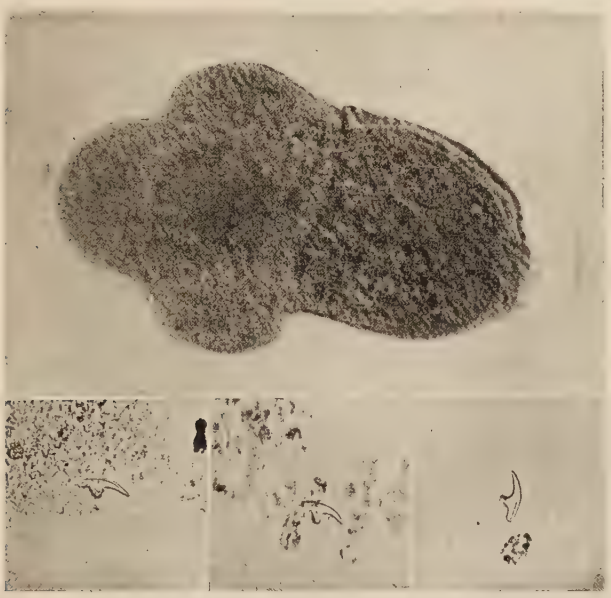
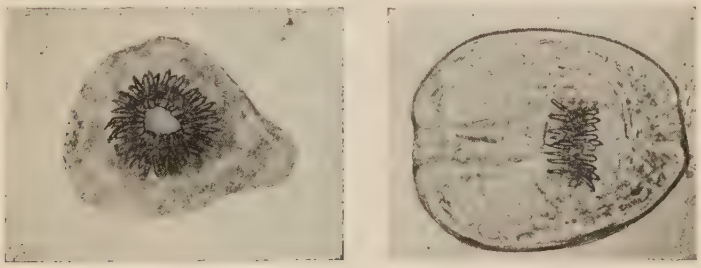


FIG. 214.—Degenerated scolex without hooklets and free hooklets of *Tænia echinococcus* in fluid from hepatic cyst (photographs × 300).

edge of the membrane. All of these structures can be found in fluid from the cysts or, less frequently, in the sputum or the urine, when the disease involves the lung or kidney (Figs. 84, 214, and 215).



A B

FIG. 215.—Scolices of *Tænia echinococcus* from a hepatic cyst: A, Portion of a degenerated scolex showing circle of hooklets; B, a well-preserved scolex with crown of hooklets invaginated, a common appearance (photographs × 250).

In such material the scolices are usually much degenerated, and many of them have entirely lost the hooklets. The scolices and

hooklets are readily found with the 16-mm. objective. The latter require a high power eye-piece, owing to their pale color and small size; but, when they are found, their appearance is striking and characteristic (Fig. 214). Their length is about 25 to 40 μ.

The statistics of Vegas and Cranwell, also of Greenway, of more than 2000 cases in South America show more than 75 per cent. localization in the liver. Magath's report of cases in the United States since 1902 showed localization in the liver in 69 cases, in the peritoneum in 10, in the lungs in 4, in the kidneys in 4, in the omentum in 4, and cysts elsewhere in 13; a total of 104 organs affected in 93 cases.

2. Genus Hymenolepis.—(1) **Hymenolepis nana,** the dwarf tapeworm (Figs. 216 and 217), is 1 to 4.5 cm. in length and 0.4 to 0.7 mm. in breadth at the widest part. The head is about 0.3 mm. broad and has a retractable rostellum with a crown of twenty-four to thirty hooklets. Its shape is generally described as globular, but depends somewhat upon the condition of the worm when killed. The segments number 150 to 200. There is no birth-pore, and the eggs escape through disintegration of the segments.

FIG. 216.—Dwarf tapeworm (*Hymenolepis nana*), adults. From photographs. Natural size.

Diagnosis must, in general, depend upon the discovery of ova in the feces, since the worms themselves are usually partly disintegrated when they leave the body and are recognized with difficulty. The ova are colorless, semitransparent, nearly spheric, and contain an embryo surrounded by two distinct membranous walls, between which is a broad zone of gelantinous substance (Fig. 218). The outer membrane is about 35 and 45 μ in its short and long diameters. The inner averages about 22 by 28 μ, and at each pole has a slight projection provided with indistinct filamentous processes, which may lie between the two membranes in such a way as sometimes to simulate a third membrane. The embryo, of which only the three pairs of hooklets are clearly seen, fills the space within the inner wall.

The worm is common in Europe and America, and is probably the most common of all the tapeworms of man in the United States. It is most frequent in children, particularly in orphanages,

and is generally present in large numbers, producing considerable digestive and nervous disturbance. Its small size, the fact that it is more or less disintegrated in the intestine, and, especially, the

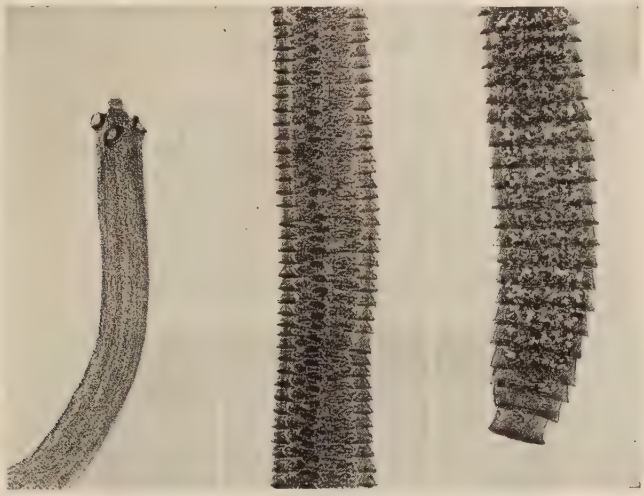


FIG. 217.—Dwarf tapeworm (*Hymenolepis nana*), head, middle segments, and termina segments. Note the protruded rostellum and the three suckers. From stained and mounted specimens (photographs × 30).

infrequency of routine microscopic examination of the feces account for the failure to recognize its prevalence in the past. Now that attention has been directed to it undigested banana fibers in the

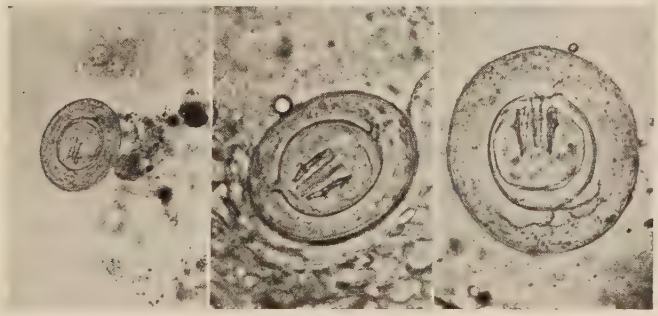


FIG. 218.—Ova of *Hymenolepis nana* in feces. The egg to the right was compressed by pressure upon the cover-glass (photographs). The figure at the left is magnified 250 diameters; the other two, 500 diameters.

diarrheal stools of children have been very frequently mistaken for it (see Fig. 179).

The mode of dissemination is not definitely known. It is pre-

sumed that the ova are transmitted to man through contamination of food by excrement of rats and mice. A similar dwarf tapeworm which is now believed to be identical with *H. nana* is a very common parasite of rats. Apparently both larval and adult stages occur in the intestine of the same host. A larval stage has also been found in certain insects.

(2) **Hymenolepis diminuta** is a common intestinal parasite of rats. A few cases of infection in man have been reported in America. The parasite measures 20 to 60 cm. in length, is very narrow, and is composed of 600 to 1300 segments. The scolex lacks hooklets. The ova resemble those of *H. nana*, but the outer shell is thicker and sometimes radially striated, and the filamentous proc-

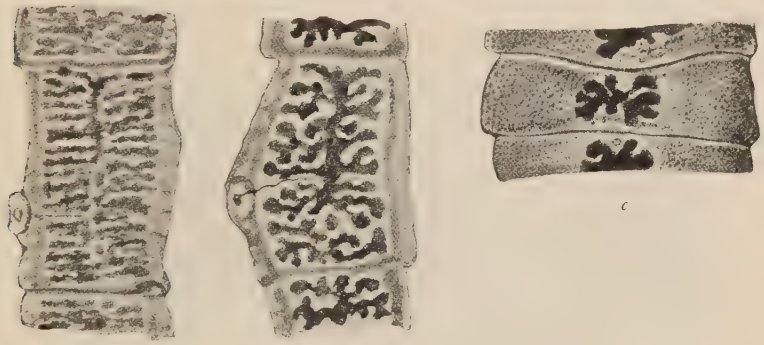
FIG. 219.—Head of the fish tapeworm, *Diphyllobothrium latum*. Above, flat side; below, edge showing characteristic groove (× 12).

esses between the two membranes are lacking. The egg is 56 to 80 μ in diameter, and the inner shell, which contains a six-hooked embryo, measures about 24 by 40 μ.

3. Genus Dipylidium.—Dipylidium caninum, sometimes called *Tænia elliptica*, is a very common tapeworm of dogs and cats. Its length is 15 to 35 cm. The head, globular in shape, is armed with hooklets. Terminal segments are shaped like cucumber seeds, 8 to 11 mm. long and 1.5 to 3 mm. broad. Ova are spheric, 43 to 50 μ in diameter, and thin-shelled. They contain a six-hooked embryo, 32 to 36 μ in diameter. The eggs are grouped in packets of eight to fifteen, and are usually passed from the bowel within the proglottides.

The intermediate host is the flea or louse. Infection of human beings is rare, and is mostly confined to children, who are probably infected from getting lice or fleas of dogs or cats into their mouths.

4. Genus Diphyllobothrium.—Diphyllobothrium latum (Dibothriocephalus latus), the fish tapeworm, sometimes reaches 12 meters in length, although it is generally not more than one-half or one-third as long. When several worms are present they are much shorter, often only 1.5 to 2 meters. The head is a flattened ovoid, about 1 mm. broad and 1.5 mm. long. It is unprovided with either suckers or hooklets, but has two longitudinal grooves which serve the same purpose (Fig. 219). The length of the segments is generally less than their breadth, mature segments



a *b* *c*

FIG. 220.—Segments of the three large tapeworms of man, showing arrangement of uterus: *a, Tænia saginata; b, Tænia solium; c, Diphyllobothrium latum* (× 5). (From P. J. Cammidge, The Feces of Children and Adults.)

measuring about 3 by 10 or 12 mm. The uterus, which is situated in the center of the segment, is roset shaped (Fig. 220, *c*) and brown or black in color. The ova are discharged through a birth-canal, and are not retained until the proglottid disintegrates, as is the case with tapeworms of the family *Tæniidæ*. The number of segments sometimes exceeds 3000. As a rule, they do not appear in the feces singly, but in chains of considerable length.

The ova are usually easily found in the feces without resort to concentration methods, although they may be scarce or absent for a short time after passage of a considerable portion of the worm. In any case the brine flotation method of concentration (p. 441) is not applicable, since operculated eggs fail to come to the surface The eggs measure about 45 by 65 μ, are brown in color, and are

filled with small spherules. The shell is thin and has a small hinged lid at one end. As the eggs appear in the feces the lid is not easily seen, but it may be demonstrated by sufficient pressure upon the cover-glass to force it open (Fig. 221). The only other operculated eggs met with in man are those of the fluke-worms. Magath has called attention to a very small projection or localized thickening of the shell at the end opposite the lid, which is helpful in distinguishing this egg from that of the flukes. This may be seen, although not clearly, in Plate XI.

When the eggs reach water a ciliated embryo develops and hatches in about two weeks. This swims about until it finds a minute crustacean, cyclops, which is the first intermediate host.

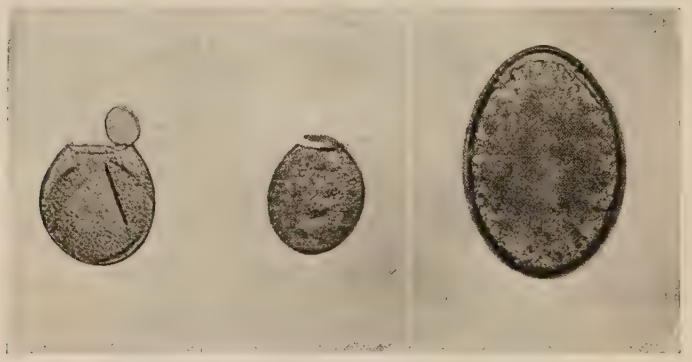


Fig. 221.—Ova of *Diphyllobothrium latum* (photographs × 250 and 500). The lids were forced open by pressure upon the cover-glass.

The crustaceans serve as food for fish, and the larvæ thus reach the second intermediate host. They do not form cysts, but live in the muscles and certain organs of the fish as worm-like structures (plerocercoids) which may grow to a length of 2 or 3 cm. These are the infective larvæ, and are found in various fish, notably the pike, burbot, grayling, and certain trout. Infection of man prevails only in regions where these fish are found. It is very common in Japan and in various countries of Europe, especially Ireland and the Baltic provinces of Russia. Many cases of infection have been reported in this country, a few of which were undoubtedly acquired here. Any locality in which cyclops and the favorable fish are native becomes a possible center of dissemination if the worm be introduced by infected immigrants. The longevity of the worm in

the intestine is difficult to determine. Riley has reported a case in which the worm existed for at least thirteen years.

Diphyllobothrium latum is interesting clinically because in many cases it causes a very severe grade of anemia, which is indistinguishable from pernicious anemia. Why it should produce anemia in some cases and not in others is not altogether clear. There is apparently some other factor than the mere presence of the worm. In a case of Todd's in which six worms with a combined length of 75 feet were present there was no appreciable anemia. The anemia is probably connected with absorption of hemolytic poisons from segments disintegrating in the intestine and may be absent when the segments are habitually passed intact. Eosinophilia may exist, but is not usual.

PHYLUM NEMATHELMINTHES

(Round Worms)

CLASS **Nematoda.**—Unsegmented, cylindric or fusiform, with alimentary tract.

Genus	*Species*
Angulilula.	*A. aceti.*
Ascaris.	*A. lumbricoides.*
Oxyuris.	*O. vermicularis.*
Filaria.	*F. bancrofti.*
	F. philippinensis.
	F. perstans.
	F. diurna.
	F. medinensis.
Ankylostoma.	*A. duodenale.*
Necator.	*N. americanus.*
Strongyloides.	*S. stercoralis.*
Trichinella.	*T. spiralis.*
Trichuris.	*T. trichiura.*

CLASS NEMATODA

The nematodes, or round-worms, are cylindric or fusiform worms, varying in length, according to species, from 1 mm. to 40 or 80 cm. As a rule, the sexes are separate, and the male is smaller and more slender than the female. In a few cases the female is viviparous; in most cases she deposits ova which are characteristic, so that the finding of a single egg may establish the diagnosis. The life-history in some cases is simple; in others complicated. It will be dealt with in the descriptions of the several species. In general,

the young are different from the adult, and must pass a certain larval stage of development before again reaching a host. An intermediate host is, however, necessary with only a few species.

The digestive tract of the nematodes is complete. The mouth is at the tip of the anterior end and is frequently surrounded by thick lips or papillæ. The esophagus is a thick muscular tube with radially arranged fibers. At the posterior end there is a bulbous expansion; or there may be a constriction in the middle with an anterior and posterior expansion (Fig. 227). The intestine is a straight thin-walled tube, ending in an anus near the tip of the posterior end of the worm.

The excretory system is drained by two lateral canals which usually unite anteriorly and end in an excretory pore on the ventral surface near the mouth. The nervous system consists of a nerve ring at the head end, a dorsal and ventral nerve cord, and circular bridges connecting the cords. Vascular and respiratory systems are lacking.

The generative organs of the male consist of a single sinuous tube which is divisible into testis, spermatic duct, seminal vesicle, and ejaculatory duct. The last opens into the terminal portion of the rectum, which is known as the cloaca. Within the cloaca are one or two spicules or copulatory organs which may be projected and retracted. The generative organs of the female usually consist of two extensively coiled tubules, each divisible into ovary, oviduct, and uterus. These unite to form a short vagina which opens upon the ventral surface of the body posteriorly or near the middle.

Between the internal organs and the ectoderm is a cavity containing lymph.

1. Genus Anguillula.—Anguillula aceti.—This worm, commonly called the "vinegar eel," is usually present in vinegar. A drop of the vinegar, particularly of the sediment, will frequently show great numbers, all in active motion: males, about 1 or 1.5 mm. long; females, somewhat larger and frequently containing several coiled embryos; and young, of all sizes up to the adult (Fig. 85).

The vinegar eel is never parasitic, but is occasionally met with as a contamination in the urine (p. 202), and has there been mistaken for the larva of filaria or strongyloides.

2. Genus Ascaris.—Ascaris lumbricoides.—The female is 20 to 40 cm. long and about 5 mm. thick; the male, from 15 to 17 cm. long and 3 mm. thick. They taper to a blunt point anteriorly and posteriorly (Fig. 222). Their color is reddish or light brown.

At the anterior tip are three small papilla-like lips which can easily be seen with a hand lens. The posterior end of the male curls ventrally, and the cloaca near the posterior tip is provided with two copulatory spicules about 2 mm. long. The vulva of the female is situated on the ventral surface at the junction of the anterior and middle thirds of the body.

The common round-worm of the hog, *Ascaris suilla*, is identical morphologically with *A. lumbricoides*, although somewhat smaller, and since it is easily obtained at slaughter-houses serves well for class study. The interesting precipitin experiments of Schwartz[1] suggest that the two

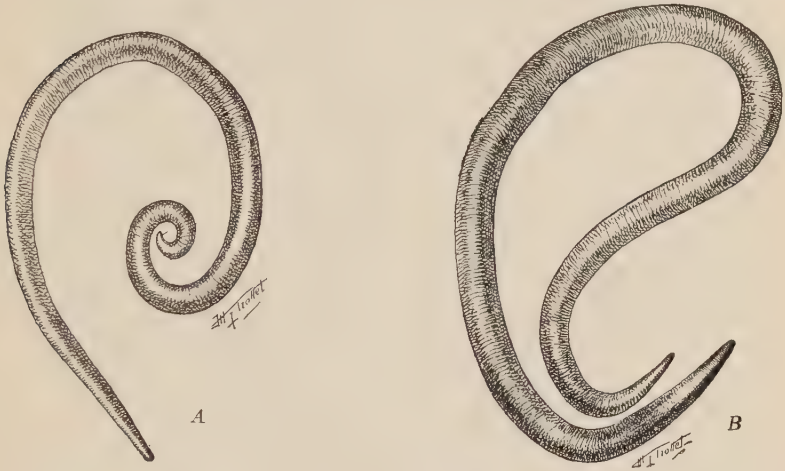


FIG. 222.—The common "round-worm," *Ascaris lumbricoides*, natural size: *A*, Male; *B*, female. (After Brumpt.)

parasites are identical biologically. The similar but much smaller ascarids so frequently present in cats (*Belascaris cati*) and dogs (*Toxascaris canis*), and commonly known as "stomach worms," have been reported some ten or twelve times in children. They produced slight or no symptoms.

Ascaris lumbricoides is the common "round-worm" so frequently found in children. Its habitat is the small intestine. Usually several individuals are present and sometimes many, even 200 or more. Symptoms—nervous and gastro-intestinal—may or may not

[1] Schwartz, B.: Biological Relationships of Ascarids, Jour. Parasitology, vol. 6, p. 115, March, 1920.

be evident. When numerous the worms may form abdominal tumors of considerable size, or even completely block the intestine. Adults are much less susceptible to infection than are children.

The diagnosis is made by detection of the worms or their ova in the feces. The worms (Fig. 222) seldom appear except as a

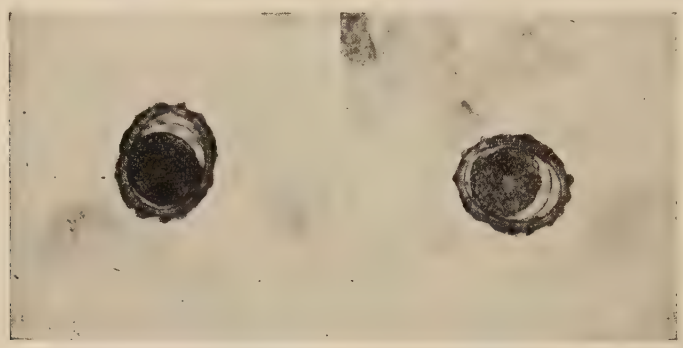


FIG. 223.—Ova of *Ascaris lumbricoides* in fresh feces (photographs × 250).

result of anthelmintic treatment. The eggs are generally numerous. As a rule, microscopic examination of the feces shows one to several upon every slide, even when no more than one laying female is present in the intestine. Typical fertilized eggs are easily recognized. They are elliptic, measuring about 45 to 50 by 60 to 75 μ,

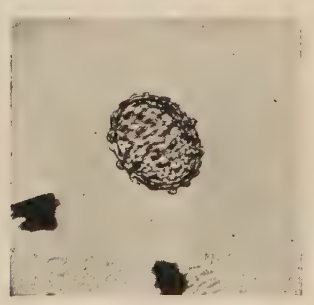


FIG. 224.—Egg of *Ascaris lumbricoides*, surface view (photograph × 250).

are yellow to brown in color, and have an unsegmented protoplasm. There is usually a crescentic clear space at each pole, between the contents and the shell (Fig. 223). The shell is moderately thick and smooth, and is covered with an irregular albuminous coating which gives it a roughly mammillated or sculptured surface (Fig. 224). Sometimes this coating is lost and the surface of the shell

is then smooth. When only females are present in the intestine, and occasionally at other times, one finds unfertilized eggs. These are generally more elongated, have a thinner shell, and are filled with coarse granular contents which obliterate the crescentic clear spaces at the ends. Many are roughly globular and some are so extremely irregular in outline (Fig. 225) as to bear little resemblance to an egg. Such unfertilized ova have doubtless many times passed unrecognized even by clinical microscopists of some experience.

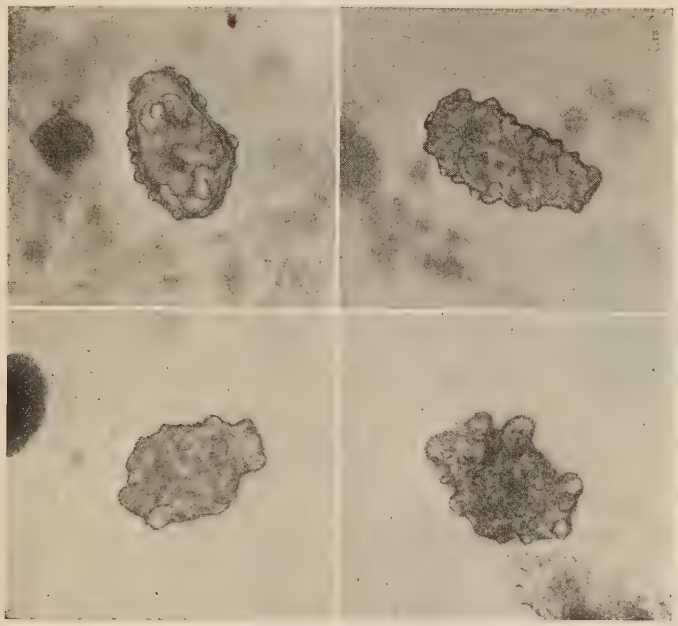


FIG. 225.—Unfertilized ova of *Ascaris lumbricoides*, showing the great irregularity in shape. Some of these eggs are extremely difficult to identify (photographs × 250).

The life-history of *A. lumbricoides* has recently been worked out in detail by Stewart, Ransom and Foster, and Yoshida, and is more complicated than was formerly supposed. The eggs pass out of the host's intestine in the unsegmented form described above. Segmentation of the germ-cell and development of the embryo take place in the open. Usually by the end of thirty days, not less than eighteen days even under most favorable conditions, the egg comes to contain a worm-like embryo, which remains without further development until taken into the intestine of an appropriate host.

The eggs are extremely resistant and in the temperate zone may pass

the winter without injury. Formaldehyd and certain other germicides have little injurious effect: apparently they harden the albuminous envelope and fail to penetrate. In the 5 per cent. formalin used for preserving feces for class demonstration many of the eggs continue slowly to segment, and some fully mature and remain alive even for a year or more. Phenol and the cresol preparations are most effective in destroying the eggs.

Unripe eggs are not infective. If ingested they pass harmlessly through the intestine. When, upon the other hand, matured eggs are ingested, the embryos leave the shells within twelve to eighteen hours. They then migrate to the lungs chiefly by way of the blood-vessels, but also by boring through the tissues, piercing the intestinal wall and the diaphragm in their course, and penetrating into the lung from the pleural cavity. In experimental animals stray larvæ can be found in the peritoneal cavity, and in the liver, spleen, and other abdominal organs. During migration the larvæ undergo a process of development and grow from a length of 0.25 mm. when in the intestine to 1.5 or 2 mm. in the lung. Finally they appear in the trachea, migrate (or are carried in mucus) to the mouth, thence down the esophagus to the stomach, and on to the small intestine, where each grows into the familiar adult ascarid. In experimental animals the cycle of migration, from the intestine and back to it again, is completed in eight days or more. The abdominal organs are apparently not injured by the passage of the larvæ, but the lungs show considerable hemorrhage. This has been suggested by Ransom as a possible important cause of pulmonary disease, even pneumonia, in children.

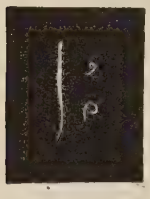


Fig. 226.—*Oxyuris vermicularis*, male and female, natural size (after Heller).

3. Genus Oxyuris.—Oxyuris vermicularis.—This is the well-known "thread-worm" or "pin-worm" which matures in the small intestine and cecum and in the adult stage inhabits the colon and rectum, especially of young children. Its presence should be suspected in all unexplained cases of pruritus ani. The female is about 9 to 12 mm. long; the male, about 3 to 5 mm. (Fig. 226).

The cuticle is transversely striated. At each side of the head is a thin, transversely striated, cuticular expansion, which is usually prominent. The bulbous esophagus can generally be clearly seen. The posterior end of the male is curled ventrally and near the tip is provided with a single copulatory spicule. The posterior end of

the female tapers to form a straight, sharply pointed tail. The vulva is situated at about the junction of the anterior and middle thirds of the body.

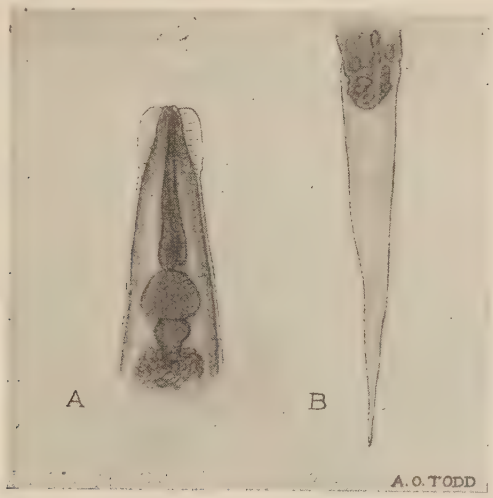


FIG. 227.—Head and tail of female "pin-worm," *Oxyuris vermicularis:* A, Head, showing the two cuticular appendages, and the beginning of the esophagus with its bulbous expansions; B, tail, showing sharply pointed tip (× 35). Compare with Fig. 233.

The worms are not infrequently found in the feces, particularly after a copious enema; the ova, rarely. The latter are best found by scraping the skin with a dull knife at the margin of the anus, where they are deposited by the female, who wanders out from the

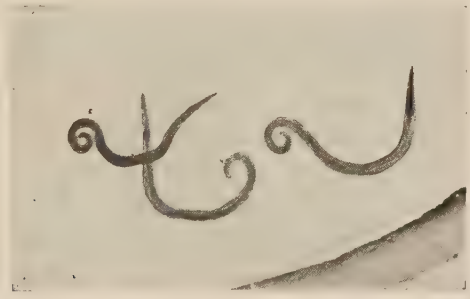


FIG. 228.—Three male pin-worms (*Oxyuris vermicularis*). Note the curled tail (photograph × 10)

rectum for this purpose, thus producing the troublesome itching. They are colorless and asymmetrically oval with one flattened side, are about 50 μ long by 16 to 25 μ wide, have a moderately thin, double-contoured shell, and when deposited contain a partially

developed embryo (Fig. 229 and Plate XI). The diagnosis is best made by giving a purgative or a copious enema and searching the stool for the adult worms. It is essential to examine the stool by the method given on page 421, best in a large Petri dish over a dark background placed some distance below. Unless the water be perfectly clear the very small male pin-worms are almost certain to be overlooked.

Infection takes place through swallowing the ova. Auto-infection is likely to occur in children; the ova cling to the fingers after scratching and are thus carried to the mouth. This is the greatest hindrance to successful treatment. Diagnosis can sometimes be made by finding the ova in the dirt beneath the finger-nails.

Kofoid and White have reported the egg of an unknown nematode evidently related to *Oxyuris vermicularis* in the feces of 429 recruits among 140,000 examined at Camp Travis, Texas. These

a *b*

FIG. 229.—Eggs of *Oxyuris vermicularis: a*, Freshly deposited, with tadpole-like embryo, *b*, twelve hours after deposition, with nematode-like embryo (× 500). (After Fantham, Stephens; and Theobald.)

men came from 22 states. Adults or larvæ were not seen, and the name *Oxyuris incognita* was tentatively given. Sandground more recently showed that these ova are those of the free-living root nematode, *Heterodera radicola*. These ova may be ingested with vegetable salads, and pass unchanged through the alimentary tract. They are larger than those of any known human intestinal nematode, ranging up to 68 by 133 μ, and averaging about 40 by 95 μ.

4. Genus Filaria.—(1) **Filaria bancrofti.**—The adults are thread-like worms, the male about 4 cm., the female about 8 cm. long. They live in pairs in the lymphatic channels and glands, especially those of the pelvis and groin, and often occur in such numbers as to obstruct the flow of lymph. This is the most common cause of elephantiasis and chyluria. Infection is very common in tropical and subtropical countries, where in some regions as high as 50 per cent. of the natives harbor microfilariæ. Even as far

north as Charleston, S. C., Johnson has found over 19 per cent. of infection among the poorer classes. Of these, only one-fourth showed any symptoms referable to the filariæ. Surveys in other parts of the Southern States have shown much lower percentages of infection.

The female is viviparous, and produces vast numbers of larvæ, which appear in the circulating blood. These are conveniently called microfilariæ; the name *Filaria sanguinis hominis*, which was formerly applied to them, is incorrect, since they do not constitute a species. These larvæ are slender, being about as wide as a red corpuscle and 0.2 to 0.4 mm. long (Fig. 155), and are very active, although, owing to the fact that they are inclosed in a loose transparent sheath, they do not move about from place to place. They are found in the peripheral blood chiefly at night, being usually easily demonstrable by 8 o'clock and reaching their maximum number—which may be enormous—about 2 A. M. By the concentration method given on page 320 a few can usually be demonstrated during the day. In the case of a medical student from Porto Rico with no symptoms Smith and Rivas found 30 microfilariæ in each cubic centimeter of blood at 4 P. M., and 6500 in each cubic centimeter at 2 A. M. If the patient change his time of sleeping, they will appear during the day. The periodicity is apparently dependent primarily upon the state of the capillaries and secondarily upon the motility of the species.

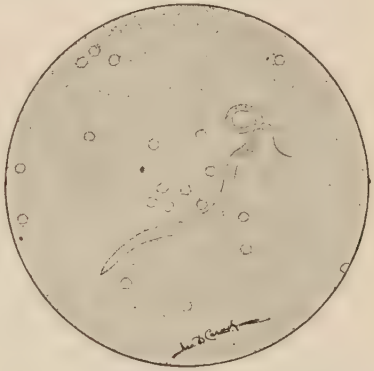


FIG. 230.—Larva of *Filaria bancrofti* in chylous hydrocele fluid; length, 300 μ; width, 8 μ. The very transparent sheath is not shown. A number of red blood-corpuscles also appear (studied through courtesy of Dr. S. D. Van Meter).

Infection is carried by certain species of mosquito, mostly belonging to the genus *Culex*, which act as intermediate host.

Diagnosis rests upon detection of larvæ in the blood, as described on page 319, but the number of larvæ found bears little relation to the severity of the symptoms, since the symptoms are largely mechanical and depend upon the localization of the adults within the body.

The larvæ are sometimes found in urine and in chylous fluids from the serous cavities. Their motion is then usually less active than when in blood. That shown in Figure 230 was alive sixty hours after removal of the fluid. Larvæ were present in the blood of the same patient.

A number of other filariæ whose larvæ appear in the blood are known, some of them only in the larval stage. Among these are *Filaria philippinensis* and *F. perstans*, which exhibit no periodicity, and *F. loa*, whose larvæ appear in the blood during the day. The adult of the last named is especially frequent in the orbit and beneath the conjunctiva.

(2) **Filaria medinensis,** the "guinea-worm," is a very interesting and important worm of Africa and Southern Asia. It has been thought to be the "fiery serpent" which molested the Children of Israel in the Wilderness.

The larva probably enters the body through the skin or gastro-intestinal tract. It wanders about in the subcutaneous tissues until maturity, producing slight, if any, symptoms. The male, which is very rarely seen, is only about 4 cm. long. It dies soon after the female is impregnated. The adult female is a very slender, yellowish worm, from 50 to 80 cm. long, its appearance somewhat suggesting a catgut suture. When gestation is complete the greater part of the female's body consists of a uterus filled with embryos. The female then travels to the feet or ankles of the host and there causes the formation of a red nodule, and, finally, an ulcer, from the center of which her head protrudes. Through this great numbers of larvæ are discharged whenever it comes in contact with water. Little damage is done unless the worm is pulled out, when the larvæ are set free in the tissues and cause serious disturbances.

When discharged the larvæ seek out a small crustacean, cyclops, which serves as intermediate host.

5. Ankylostoma duodenale and Necator americanus.— These, the Old and New World hookworms, respectively, are among the more harmful of the animal parasites. They inhabit the small intestine often in great numbers, and commonly produce an anemia which is often severe and sometimes fatal. The presence of a few, however, may cause no appreciable disturbance.

The anemia is probably due to (*a*), abstraction of blood by the worms and hemorrhage from the bleeding points left when the

worms change position in the intestine, the continued bleeding being due to a secretion of the buccal glands which hinders coagulation; (*b*) toxic secretions of the parasites, and (*c*) secondary microbic infection. In well-marked cases the red cells average 2,500,000 to 3,000,000 in each cubic millimeter, hemoglobin 35 to 50 per cent., color index low. Eosinophilia of 10 to 25 per cent. is the rule, but may be absent. Charcot-Leyden crystals are often present in the feces.

Ankylostoma duodenale is common in southern Europe and in Egypt, and is not infrequently found in America.

FIG. 231.—Hookworm (*Ankylostoma duodenale*), life size. Shows some worms adherent to a bit of intestinal mucosa and some free (from Jefferys and Maxwell).

FIG. 232.—Heads of hookworms showing mouth parts: *A, Necator americanus; B, Ankylostoma duodenale.* (After E. R. Stitt.) As the head of the hookworm is sharply curved backward, the upper part of the figure represents the ventral surface.

The body is cylindric, reddish in color, and the head is bent sharply dorsally.

There is a well-marked buccal cavity which carries three pairs of hook-like teeth, two pairs situated on the ventral side and one pair dorsally (Fig. 232). The female is 12 to 15 mm. long, and the tail is bluntly pointed. The vulva is situated at the junction of the middle and posterior thirds of the body. The male is 8 to 10 mm. long and the posterior end is expanded into an umbrella-like pouch, the caudal or copulatory bursa, which is supported by a number of stiff ribs or "rays" whose mode of branching is helpful in determining the species. Through the opening of this bursa project (unless retracted) two very slender hair-like copulatory spicules which can be seen only with a lens (Fig. 233).

The eggs are oval and have a thin, smooth, transparent shell. As they appear in the feces the protoplasm is divided into 2, 4, 8, or more rounded segments. They measure 32 to 38 by 52 to 61 μ.

Necator americanus is very common in central and southern Africa and in subtropical America, including the southern part of the United States and the West Indies Extensive surveys among rural school children by the plain smear method in eleven southern states in 1920–1923 showed 27.8 per cent. infected. In Porto Rica 90 per cent. of the rural population was infected until active measures were taken to combat the disease. Isolated cases, probably imported, have been seen in most of the northern states.

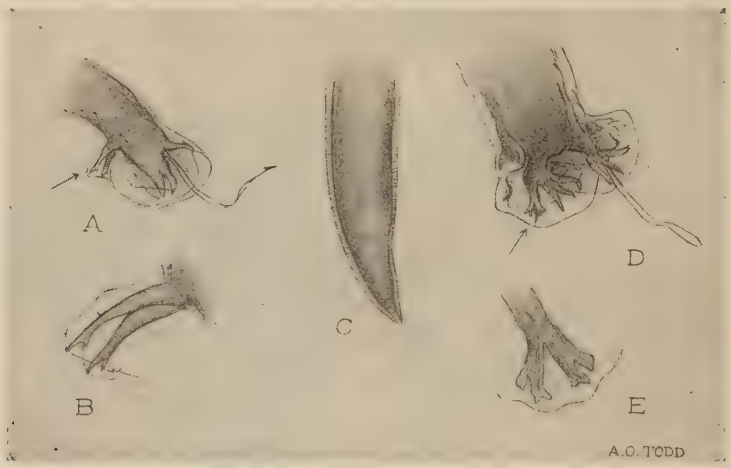


FIG. 233.—Tails of hookworms: *A*, *Necator americanus*, male, showing copulatory bursa and spicules with barb (× 35); *B*, enlarged drawing of the rays indicated by an arrow in *A*; *C*, tail of female (× 35); *D*, *Ankylostoma duodenale*, male, showing copulatory bursa and spicules without barb (× 35); *E*, enlarged drawing of the rays indicated by an arrow in *D*. (*A*, *B*, *D*, *E* after E. R. Stitt.)

Necator americanus is smaller than *A. duodenale*, the male being 7 to 9 mm. long, the female 9 to 11 mm. The four ventral hook-like teeth are replaced by two well-developed semilunar chitinous plates, and the dorsal pair of teeth by two poorly developed plates (Fig. 232). Within the buccal cavity, projecting from the dorsal wall, is a prominent, conical, tooth-like structure which forms the outlet for the head gland, and is more prominent than is its analogue in *A. duodenale*. The caudal bursa of the male is similar to that of *A. duodenale*, but there is a different arrangement of the rays. The copulatory spicules unite at the tips into a barbed point, which when seen is very helpful in determining the species (Fig. 233). The vulva of the female is situated anterior to the

middle of the body, and can readily be located with a low power of the microscope.

The ova (Fig. 234) very closely resemble those of *Ankylostoma duodenale*, but are larger, 36 to 45 by 64 to 76 μ.

The life-history of the two species is probably the same. The ova pass out with the feces, and, under favorable conditions of warmth and moisture, develop an embryo which hatches within

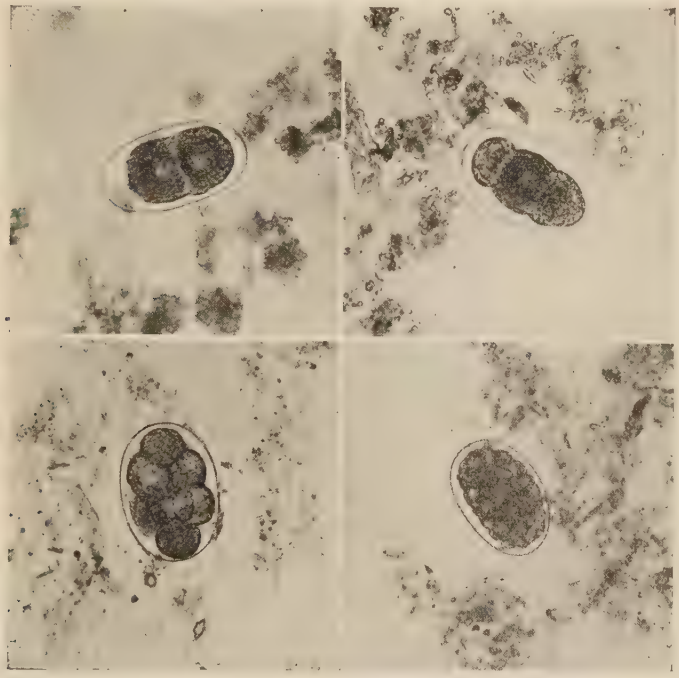


FIG. 234.—Ova of *Necator americanus* in feces. The egg, showing three cells, is a lateral view of a four-cell stage (photographs × 250).

a few days. The resulting larvæ pass through a stage of development in warm moist earth, moulting twice, and growing to a length of 0.5 to 0.6 mm. They are then ready to infect a new host. Cort and Payne found that their life in the soil does not exceed three to six weeks, and that they do not migrate more than 4 inches from the spot where they are deposited. In some cases they probably reach the host's intestine by way of the mouth, with food or water, but the usual route is probably by way of the skin, as established by Looss. When moist earth containing the larvæ comes in con-

tact with the skin they penetrate into the subcutaneous tissues. This is favored by retention of mud between the toes of those who go barefooted. When the larvæ are abundant a dermatitis is induced ("ground itch"). From the subcutaneous tissue they pass by way of lymph- and blood-streams to the lungs. Here they make their way into the smaller bronchi, are carried by the bronchial mucus to the pharynx, and are swallowed. They thus ultimately reach the small intestine, where they develop into mature worms.

The diagnosis of hookworm infection, which is assuming increasing importance in this country, usually rests upon detection of ova in the feces. The worms themselves seldom appear except after a vermifuge. A small portion of the feces, diluted with water if necessary, is placed upon a slide and the larger particles removed. The material is covered and searched with a 16-mm. objective. A higher power may rarely be necessary to positively identify an egg, but should not be used as a finder. The eggs (Fig. 234 and Plate XI) are nearly always typical, showing a thin, but very distinct shell, a clear zone, and a finely granular segmented protoplasm. A light spot, representing the nucleus, can usually be made out in each segment. After having once been seen the eggs are not easily mistaken. For their measurements see pages 506 and 507.

In heavy infections they may be found in nearly every microscopic field; in most cases, even when so mild as to cause no symptoms, they can be found on the first slide examined. It is seldom necessary to search more than half a dozen slides. From the estimate of Dock and Bass, it seems probable that ova will average at least one to the slide if ten or more laying females are present in the intestine. Very old females may fail to produce eggs. When they are scarce, some method of sedimenting the feces should be tried (p. 441).

Pepper's method of concentration is simple, but is not applicable to other ova than those of the hookworm. It is best first to sediment the feces. A layer of the diluted feces is placed on a slide and allowed to remain for some minutes. The slide is then gently immersed in water. The ova, which have settled to the bottom, cling to the glass and are not washed away as is other material. This may be repeated several times and numerous eggs collected.

Stoll's method of counting eggs in feces is recommended by Cort as the only accurate means of compiling statistical evidence in preliminary surveys, evaluating effects of treatment, or measuring the results of control measures. The technic is as follows:

1. Feces in their container are balanced on the scales together with a spatula, and 3 gm. weighed, by difference, into a large-sized test-tube or centrifuge tube graduated at 45 c.c.

2. Decinormal NaOH is poured in to the 45 c.c. mark.

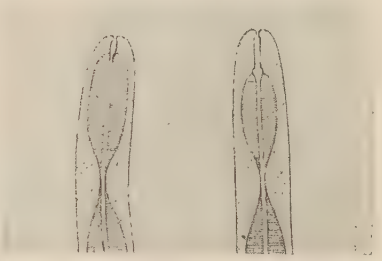
3. Ten small (3 mm.) glass beads are added, the tube rubber-stoppered, and the mixture vigorously shaken for one minute.

4. Exactly 0.15 c.c. is pipeted with an accurate pipet on to a 2 x 3-inch slide, and covered with a 22 x 40-mm. No. 2 cover-glass.

5. A mechanical stage is used and all the eggs on a slide are counted with low power.

6. The number of eggs in each gram of feces is found by multiplying the count by 100.

Two counts are made from different 3 gm. samples and averaged. The diluted material must be removed from the tube immediately after shaking.



A B

Fig. 235.—Diagram showing the difference in length of the mouth cavities of the larvæ of A, *Strongyloides*, and B, *Necator*. The heavily shaded portions represent the bulbous esophagus.

Hookworm larvæ are not found in fresh feces, but may hatch within twenty-four to forty-eight hours after the stool is passed. They are then easily mistaken for the larvæ of *Strongyloides stercoralis*, but can be distinguished by the depth of the mouth cavity, which is about equal to the diameter of the larva at the posterior end of the cavity. In *St. stercoralis* the mouth is about one-half as deep (Fig. 235). The size of the genital anlage is also important (p. 511).

Cultivation of the larvæ may be resorted to for diagnosis, and is said to be more effective than the search for eggs even by concentration methods. The Baermann apparatus as modified by Cort[1] and his co-workers is especially convenient. This consists of an ordinary 8-inch glass funnel supported on a ring stand. The tip of

[1] Cort, W. W., Ackert, J. E., Augustine, D. L., and Payne, Florence K.: The Description of an Apparatus for Isolating Infective Hookworm Larvæ from Soil, Amer. Jour. Hyg., vol. 2, p. 1, January, 1922.

the funnel is covered with a short piece of rubber tubing which is closed by pinching tightly with a Hoffman clamp. The funnel is nearly filled with water heated to 115° F., and a piece of wire-screen is so placed that it dips below the surface of the water. Over this and dipping just below the surface of the water is placed a small towel. In the center of the cloth and in the pool of water is placed the infested soil or feces. The larvæ grow out in a few days at room temperature and sink to the tip of the funnel. They can be readily drawn off in a drop of water on to a microscope slide by slightly loosening the Hoffman clamp, or a few cubic centimeters may be drawn off into a centrifuge tube and the larvæ sedimented by centrifugalization.

FIG. 236.—*A*, Egg of *Strongyloides stercoralis* (parasitic mother worm) found in stools of a case of chronic diarrhea; *B*, rhabditiform larva of *Strongyloides stercoralis* from the stools. (William Sydney Thayer in Journal of Experimental Medicine.)

Occult blood can always be detected in the feces when hook-worms are present in the intestine in any considerable number.

6. Genus Strongyloides.—Strongyloides stercoralis.—Infection with this worm is by no means so rare in this country as the few clinical reports would indicate. It is apparently wide-spread in the southern states, and is very common in subtropical countries, notably in Italy and in southern China. It is possible that the parasite may cause a mild catarrhal enteritis, although most authorities regard it as harmless. It is extremely resistant to treatment.

The adult worm, which is probably a hermaphrodite, in which the male organs atrophy early or possibly a female reproducing by parthenogenesis, is about 2 mm. long, and inhabits the upper por-

tion of the small intestine, but neither it nor the ova appear in the stool unless an active diarrhea exists. Ordinarily ova hatch in the intestines, and when infection is heavy larvæ can be found in the feces in enormous numbers. They have also been found in the duodenum. These are the "rhabditiform larvæ," which measure 250 to 500 μ by 15 to 24 μ, according to their age. Just back of the middle of the body is a conspicuous oval structure, the genital anlage, about 30 μ long (Fig. 236). This and the length of the mouth cavity (p. 509) are important in distinguishing these larvæ from those of the hookworm in which the genital anlage is very small and inconspicuous. These larvæ are actively motile, with a striking "wriggling" motion, and, when the stool is solid, are best found by making a small depression in the fecal mass, filling it with

FIG. 237.—Rhabditiform larva of *Strongyloides stercoralis* in feces (photograph × 150).

water, and keeping in a warm place (preferably an incubator) for twelve to twenty-four hours. The larvæ will collect in the water, and can be easily found by transferring a drop to a slide and examining with a 16-mm. objective. The inexperienced worker should *make sure that the worms move*, or he may be misled by the vegetable hairs which are generally present in the feces. Certain of these hairs (notably those from the skin of a peach) closely resemble small worms (p. 431).

Outside the body the rhabditiform larvæ develop into a free-living, sexually differential generation. The young of this generation are the more slender "filariform larvæ" which constitute the infective form. Direct transformation of rhabditiform into filariform larvæ also occurs. Infection takes place by ingestion or by way of the skin.

Sandground has reported the finding of *Rhabditis hominis* Kobayashi in stools sent to him for examination supposedly containing *Strongyloides stercoralis*. This worm, which greatly resembles *S. stercoralis*, is not a parasite, but a free-living coprophagous species that may be introduced into the feces, after leaving the body, by contamination with soil, or by filth flies.

7. Genus Trichinella.—Trichinella spiralis.—This is a very small worm—adult males, 1.5 to 1.6 by 0.04 mm.; females, 3 to 4 by 0.06 mm. Infection in man occurs from eating of pork which contains encysted larvæ and is insufficiently cooked. Ordinary "curing" of pork does not kill them. According to Winn heating to 55° C. for fifteen minutes for each pound of meat is sufficient to kill all larvæ. Six days' refrigeration at 0° F. (−17.7° C.) is also effective. Protection against infection must be secured through such measures as these; meat inspection is of little value unless every part of the carcass be examined, and this, of course, is impracticable. When the larvæ reach the stomach the capsule surrounding them is digested away, and they grow to maturity in the small intestine. Soon after copulation the males die, and the females penetrate into the mucous membrane, where they live for about six weeks, giving birth to great numbers of young, averaging as high as 1500 from a single female. The larvæ migrate to the striated muscles, chiefly near the tendinous insertions, where they grow to a length of about 0.8 mm., and finally become encysted. In this condition they may remain alive and capable of development for twelve years or longer.

Trichinella is wide-spread throughout the world and is more abundant in the United States than the reported cases of human infection would lead one to expect. It is capable of living in many animals, but is most common in the pig and the rat. Rats when once infected continue the infection through cannibalism. A convenient means of finding whether the parasite is common in a given community is to examine a series of slaughter-house rats.

Excepting during the acute stage trichiniasis is generally accompanied by a marked eosinophilia (p. 296). The diagnosis is made by teasing out upon a slide a bit of muscle, obtained in man preferably from the pectoralis major, the outer head of the gastrocnemius, the insertion of the deltoid, or the lower portion of the

biceps. In the case of rats the diaphragm, which is the most likely site, is pressed out between two glass slides. The coiled larvæ can easily be seen with a 16-mm. objective (Fig. 238). The larvæ can usually be found in the spinal fluid and the blood (p. 320) before they have reached their final resting-place in the muscles. During the diarrheal stage adults may be present in the feces, and can

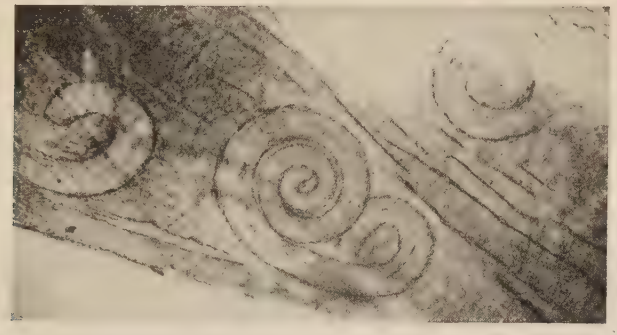


FIG. 238.—*Trichinella spiralis* in a bit of human muscle teased and mounted upon a slide (photograph with diaphragm nearly closed, × 125).

sometimes be found by diluting with water, decanting several times, and examining the sediment in a very thin layer in clean water with a hand lens.

8. Genus Trichuris.—Trichuris trichiura (Trichocephalus dispar).—This, the "whip-worm," is 3.5 to 5 cm. long. Its anterior portion is slender and thread-like, while the posterior portion is

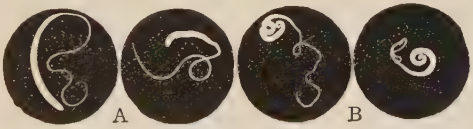


FIG. 239.—Whip-worms (*Trichuris trichiura*): A, Females; B, males. The posterior portion of the male is usually coiled as is shown at the right. Photographs of mounted specimens. Natural size.

thicker (Fig. 239). It is widely distributed geographically, and is one of the most common of intestinal parasites in this country. It lives in the large intestine, especially the cecum, with its slender extremity embedded in the mucous membrane. Whip-worms do not, as a rule, produce any symptoms, although gastro-intestinal disturbances, nervous symptoms, and anemia have at times been ascribed to them. They, as well as many other intestinal parasites,

are probably an important factor in the etiology of appendicitis, typhoid fever, and other intestinal infections. The damage which they do to the mucous membrane favors bacterial invasion. They are extremely refractory to anthelmintic treatment.

The number present is usually small. The worms themselves are rarely found in the feces. The ova, which are not often abundant, are easily recognized with the 16-mm. objective. Although they are comparatively small, their appearance is striking. They are brown, ovoid in shape, 50 to 54 μ long by about 23 μ wide, and have a button-like projection at each end (Fig. 240).

The eggs are said (Castellani) to require eighteen months in the open before the embryo is fully developed. Not until then are

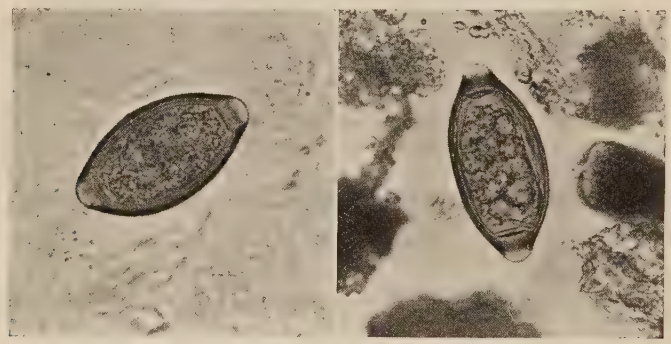


FIG. 240.—Ova of *Trichuris trichiura* in feces (photographs × 500).

they infective. They reach the host through contaminated food and water.

PHYLUM ARTHROPODA

The arthropoda are of medical interest chiefly because they are among the most important agents for the spread of disease. Many are simple carriers and disseminators of bacteria, as fleas carry plague bacilli, and flies, typhoid bacilli. Others serve as the necessary hosts for a part of the life-cycle of important animal parasites of which they are thus the sole "vectors," as certain mosquitoes are the vectors of the malarial parasites.

Only a few of the arthropoda are directly parasitic, and these are unimportant, so that only brief descriptions need be given in the following pages. They belong to the classes *Arachnida* and *Insecta*.

Class Arachnida

The class includes spiders, mites, and ticks. In general the body is divided into two regions, cephalothorax and abdomen. Antennæ are absent. The two orders to which the parasitic forms belong have become considerably modified from the type. In the order *Acarina*, comprising mites and ticks, "the body is an unsegmented sac to which is attached a movable capitulum, or false head, bearing the mouth parts" (Root). In the order *Linguatulida* or "tongue worms" the body is ringed, elongated and worm-like, and legs are absent in the adult.

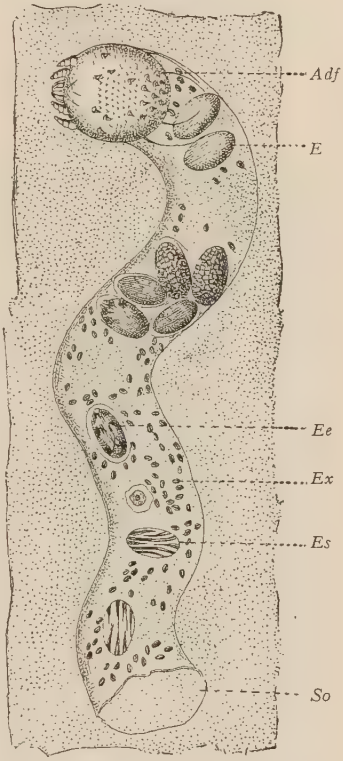


Fig. 241.—*Sarcoptes scabiei.* Diagram of a subcutaneous burrow: *Adf*, Adult female; *E*, eggs; *Ee*, embryo egg; *Ex*, excrement; *Es*, egg shell; *So*, skin orifice. (After Railliet in Brumpt.)

The most important of the parasitic mites is the well-known "itch mite," *Sarcoptes scabiei*. Its tunnels in the skin can be seen with a good magnifying glass as lines 2 to 4 mm. long, often black from the presence of dirt or the excrement and eggs of the parasite (Fig. 241). The parasite and its eggs can often be found by scraping the burrow with a small scalpel, and mounting the scrapings in water on a slide. To see them well the low power of the microscope is required. The parasite is round or oval and set with bristles (Fig. 242). The males average about 170 by 220 μ, females about 300 by 400 μ. Related species cause mange in dogs and cats, and may very rarely attack man.

Another mite, *Demodex folliculorum*, the "face insect," lives in the hair-follicles and sebaceous glands of a large proportion of human beings, where it produces no disturbance beyond mild irritation. The parasites can be found by pressing out the contents of the glands and examining the material with a low power of the microscope by very subdued light (Fig. 243). The male measures

about 40 by 300 μ, while the female is a little longer. A related parasite causes an obstinate type of mange in dogs.

There are a number of mites, the six-legged larvæ of which parasitize insects and small animals ordinarily, but which, when the

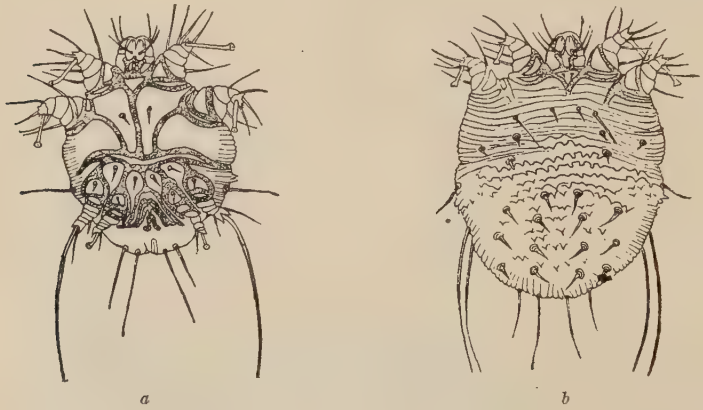


FIG. 242.—The itch mite, *Sarcoptes scabiei: a*, Male; *b*, female (× 100). (Riley and Johannsen, after Fürstenberg.)

opportunity offers, may attack man, burrowing into the skin or entering the sweat-glands, and thus causing inflammation and intense itching. These are very common in some parts of the

FIG. 243.—The "face insect" *Demodex folliculorum* (× 100); *Kt.*, Biting jaws. (After R. Blanchard in Brumpt.)

FIG. 244.—The spotted fever tick, *Dermacentor andersoni*, male (left) and unengorged female (right) (× 3½). (From Herms' Medical and Veterinary Entomology, courtesy of the Macmillan Company, Publishers.)

United States, and are popularly known as mites, red bugs, chiggers, and so forth. Most of them are just visible to the unaided eye.

In many regions the larger members of the order *Acarina*, the

ticks, are a source of annoyance, but they are of little medical importance, save as agents for the transmission of such diseases as relapsing fever and Rocky Mountain spotted fever. Rocky Mountain fever is carried chiefly by the wood-tick, *Dermacentor andersoni* (Fig. 244). This same tick, as well as certain insects, may transmit the newly discovered disease tularemia.

One other parasitic arachnid, *Linguatula serrata*, the "tongue worm," requires mention since a few cases of human infection have

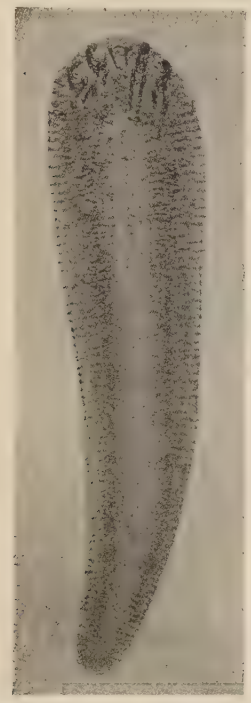


FIG. 245.—Larva of *Linguatula serrata* (de Faria and Travassos).

FIG. 246.—Body louse (*Pediculus vestimenti*), female (× 15). The male is a little smaller and the posterior end of the abdomen has no notch. The body louse is distinguished from the head louse by its larger size and by the relative widths of thorax and abdomen.

been reported from Europe, the Panama Canal Zone, and Brazil; it may prove to be more common than has been recognized. This and related species are not rare in dogs, horses, goats, and certain birds. Man may be parasitized by either the adult or larval stage.

The adults are worm-like, without legs, and live in the nasal passages attached to the mucous membrane. The female, which is several times as large as the male, attains a length of 8 to 10 cm. The eggs are carried out with the nasal discharges and ultimately

reach another host where the larvæ hatch out and soon encyst in the internal organs, particularly the liver. After a period of development the larvæ again become free, and in this stage usually reach the feces, where, because of their serrations, they may be mistaken for minute tapeworms (Fig. 245). They are white and measure about 4 to 6.5 mm. long and 0.9 to 1.5 mm. broad at the widest (anterior) part.

Class Insecta

In adult members of this class the body is divided into three distinct regions, head, thorax, and abdomen; and the head bears one pair of antennæ. There are three pairs of legs, all attached to the thorax. Typically there are one or two pairs of wings, but in most parasitic species the wings have been lost with the adoption

Fig. 247.—Head louse (*Pediculus capitis*), male (× 15). The female is a little larger and the posterior end of the abdomen is notched.

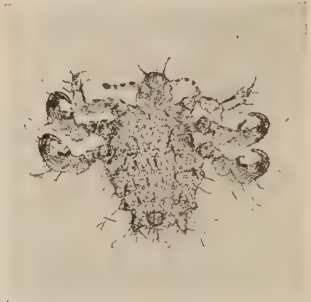


Fig. 248.—Pubic louse (*Phthirius pubis*) (× 15).

of the parasitic mode of life. Parasitic insects include lice, fleas, and the larvæ of certain flies.

The structure of the fleas, lice, and similar small insects may be more easily studied if they are made more or less transparent by soaking them for a day or two in 20 per cent. solution of sodium hydroxid.

The lice are wingless and are flattened dorsoventrally. There are many species, all of which are closely confined to their particular host species. Only three infest man: the body louse, *Pediculus vestimenti;* the head-louse, *Pediculus capitis;* and the pubic louse, *Phthirius pubis.* These are sufficiently described by the accompanying pictures, and their legends (Figs. 246–248). The head louse and the pubic louse attach their eggs to hairs, where they are

commonly known as "nits" (Fig. 249). The body louse lives in the clothing, and seeks the body only at the time of feeding. Its eggs are cemented to the clothing, chiefly in the folds and seams.

FIG. 249.—Empty egg of *Pediculus capitis* cemented to a hair. Before hatching the egg is covered with a cap or lid (photograph × 15).

FIG. 250.—The flea, *Pulex irritans*, male. Note the absence of "combs" (photograph × 15).

The fleas are reddish brown, wingless, and flattened laterally. Their shape permits them to move rapidly along the hairs. While each species has its favorite host, the fleas are not strictly confined to particular host species as are the lice. The best-known human

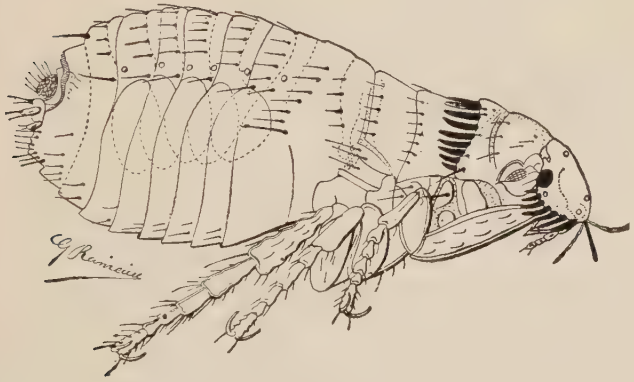


FIG. 251.—*Ctenocephalus canis*, female (× 25). (After Brumpt.) Note the conspicuous "combs" along inferior border of head and on prothorax.

FIG. 252.—Head of *Ceratophyllus*. Note "combs" only on prothorax. (After Brumpt.)

flea is *Pulex irritans* (Fig. 250). A dog-flea, *Ctenocephalus canis* (Fig. 251), also infests man in certain regions. The two chief rat fleas are *Ceratophyllus fasciatus* (Fig. 252) and *Xenopsylla cheopis*. The latter is the rat flea of India, and is, therefore, the more con-

cerned in the transmission of plague. It closely resembles *Pulex irritans*, being without combs, but is more yellow.

The fleas mentioned live on the surface of the body. A very small flea, *Tunga* (*Dermatophilus*) *penetrans*, which burrows into the skin, is common in tropical and subtropical America and Africa. This is popularly known as the chigæ flea or "chigger," but should not be confused with the larval mites which are called "chiggers" in the United States (p. 516)

A number of flies, including the common house-fly, may deposit their ova in wounds or in such of the body cavities as they can

FIG. 253.—*Chrysomyia macellaria*, screw-worm fly: *A*, Adult; *B*, maggot (× 3). (*A*, after Castellani and Chalmers; *B*, after Blanchard.)

reach, and the resulting maggots may cause intense irritation. To this condition the general term *myiasis* is applied. Ova may be swallowed with the food, and the maggots appear in the feces. In some cases ova are deposited in the tonsillar crypts, with resulting formation of abscesses. A few cases in which larvæ have been found in the urinary passages have been reported. The physician should be able to recognize these as maggots, but the exact determination of species is a matter for the trained entomologist.

Probably the most important form of myiasis is infection with the "screw worm," the larvæ of *Chrysomyia macellaria* (Fig. 253), which is not rare in some parts of the United States, particularly

west of the Mississippi. The ova are most commonly deposited in the nasal passages, and the larvæ, which may be present in great numbers, burrow through the soft parts, cartilage, and even bone, always with serious and often with fatal results.

Cutaneous myiasis, in which fly larvæ develop in the skin, producing boil-like nodules similar to "bots" or "warbles" in animals, is not rare in tropical America, and a few imported cases have

FIG. 254.—Larva of *Dermatobia hominis*. (× 3).

FIG. 255.—The common bedbug, *Cimex lectularius*, male (× 5). In the female the posterior end of the abdomen is more rounded. (Cleared with sodium hydroxid to bring out the structure more clearly.)

been seen in this country. The best known is the larva of the fly *Dermatobia hominis*, ver macaque of the tropics (Fig. 254), which is transferred to man by mosquitoes, and possibly by ticks, to which the fly affixes its eggs.

Among insects which are not parasitic, but are extremely important as vectors of disease, are mosquitoes (p. 312), the tsetse fly (p. 463), the South American house bug or "cone nose," *Triatoma*, (p. 464), and the well-known bedbug, *Cimex lectularius* (Fig. 255).

CHAPTER VII

PUS, PUNCTURE FLUIDS, ANIMAL INOCULATION

PUS

Pus contains much granular débris and numerous more or less disintegrated cells, the great majority being polymorphonuclear leukocytes—so-called "pus-corpuscles." Eosinophilic leukocytes are common in gonorrheal pus and in asthmatic sputum. Examination of pus is directed chiefly to detection of bacteria.

When very few bacteria are present cultural methods, which are outlined in Chapter X, must be resorted to. When considerable numbers are present, they can be detected and often identified in cover-glass smears. Several smears should be made, dried, and fixed, as described on page 657.

One of these should be stained with a bacterial stain. Löffler's methylene-blue and Pappenheim's pyronin-methyl-green are especially satisfactory for this purpose. These stains are applied for one-half minute to two minutes or longer, without heating; the preparation is rinsed in water, dried, mounted, and examined with an oil-immersion lens. Another smear should be stained by Gram's method (p. 657). These will give information concerning all bacteria which may be present in any considerable numbers, and frequently no other procedure will be necessary for their identification.

As a control of treatment of infected wounds it is desirable to determine approximately the number of bacteria in the pus from day to day. For this purpose a loopful of the pus or discharge from the worst-looking part of the wound is spread on a slide, dried, fixed, and stained with Löffler's methylene-blue, carbol-thionin, or other appropriate stain. The film is examined with the low power and an area selected in which the pus-cells just touch, but do not overlap. This area is then studied with the oil-immersion (1.9 mm.) objective and 10 × eye-piece. The average number of bacteria per field is counted and recorded, and the predominant type, whether streptococcus, staphylococcus, or bacillus, is noted. From 5 to 20 fields must be counted to get a reliable average. As the wound improves the number of bacteria per field should fall to 1 or 2, when, in the Carrel method of treatment, the wound may be closed.

The most common pus-producing organisms are **staphylococci** and **streptococci.** They are both cocci, or spheres, their average diameter being about 0.7 μ. Staphylococci are commonly grouped in clusters, often compared to bunches of grapes (Fig. 256). There are several varieties which can be distinguished only in cultures. Streptococci are arranged side by side, forming chains of variable length (Fig. 257). Sometimes there are only three or four individuals in a chain; sometimes a chain is so long as to extend across several microscopic fields. Streptococci are more virulent than staphylococci, and are less commonly met. Both are Gram-positive, but both may at times be relatively Gram-negative when inclosed within pus-corpuscles (degenerating forms). Their culture characteristics are given on page 663.

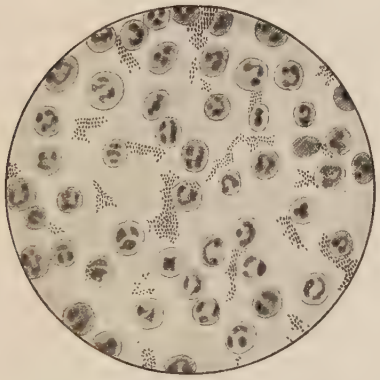


FIG. 256.—*Staphylococcus pyogenes albus* from an abscess of the parotid gland (Jakob).

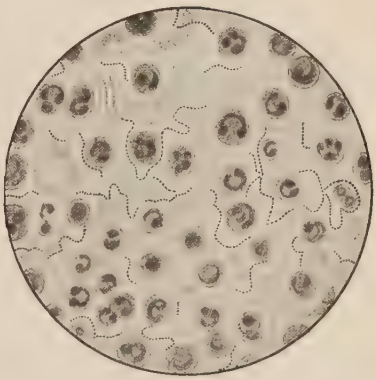


FIG. 257.—*Streptococcus pyogenes* from a case of empyema (Jakob).

Should bacteria resembling **pneumococci** be found, Rosenow's or Smith's method for capsules (p. 73) should be tried. When these are not available, capsules can usually be shown by the method of Hiss. The dried and fixed smear is covered with a stain composed of 5 c.c. saturated alcoholic solution gentian-violet and 95 c.c. distilled water, and heated until steam rises. The preparation is then washed with 20 per cent. solution of copper sulphate, dried, and mounted in Canada balsam.

Pneumococci, while most common in connection with the lung and pleural cavity, may give rise to inflammation in many locations (p. 72). When they form short chains, demonstration of the capsule or cultural methods may be necessary to distinguish them

from streptococci. The presence of a capsule is extremely significant, but is not always conclusive, since streptococci may very rarely show capsules. Cultural methods and the technic of determining the types of pneumococci are given on page 665.

If tuberculosis be suspected, the smears should be stained by one of the methods for the **tubercle bacillus** (pp. 65–67), or guinea-pigs may be inoculated. The bacilli are generally difficult to find in pus, and bacteria-free pus would suggest tuberculosis.

Gonococci when typical can usually be identified with sufficient certainty for clinical purposes in the smear stained with Löffler's methylene-blue or, much better, Pappenheim's pyronin-methyl-green. They are ovoid or coffee-bean-shaped cocci which lie in pairs with their flat surfaces together. They lie for the most part within pus-cells, an occasional cell being filled with them, while the surrounding cells contain few or none (Fig. 259). Their intracellular position and their appearance in clusters are very important points in their identification. While a few are often found outside of cells, one should hesitate to accept them as gonococci unless further search reveals intracellular organisms. It is usually difficult to find gonococci when many other bacteria are present, even though the pus is primarily of gonorrheal origin. Whenever the identify of the organism is at all questionable, Gram's method should be tried, but it should be remembered that staphylococci, streptococci, and pneumococci are sometimes relatively Gram-negative when lying intracellularly. In rare instances it may be necessary to resort to cultures. The gonococcus is distinguished by its failure to grow upon ordinary media (p. 669).

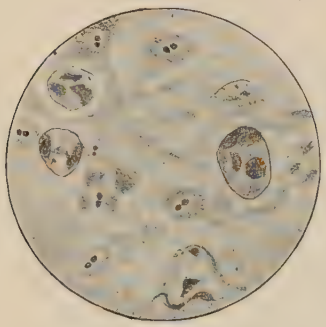


FIG. 258.—*Diplococcus pneumoniæ* from ulcer of cornea (oil-immersion objective). Study through courtesy of Dr. C. A. Oliver (Boston).

Gonococci are generally easily found in pus from untreated, acute and subacute gonorrheal inflammations—conjunctivitis, urethritis—but are found with difficulty in pus from chronic inflammations and abscesses, and in urinary sediments.

In the urine gonococci are most likely to be present in the well-known "gonorrheal threads" or "floaters," which consist of

strands of mucus with entangled pus-corpuscles, and are suggestive of chronic gonorrhea, but are by no means diagnostic of it. These are fished out with a platinum wire, spread upon slides, fixed, and stained. When floaters are absent it may be necessary to examine the sediment obtained by thorough centrifugation. In order to remove urea, which prevents proper drying of the smear, the sediment may be washed once with water or physiologic saline. Smears should be thin and quickly dried in order that the pus-corpuscles may be as well preserved as possible. Very often the pus-cells are so shrunken that the contained gonococci are difficult to recognize.

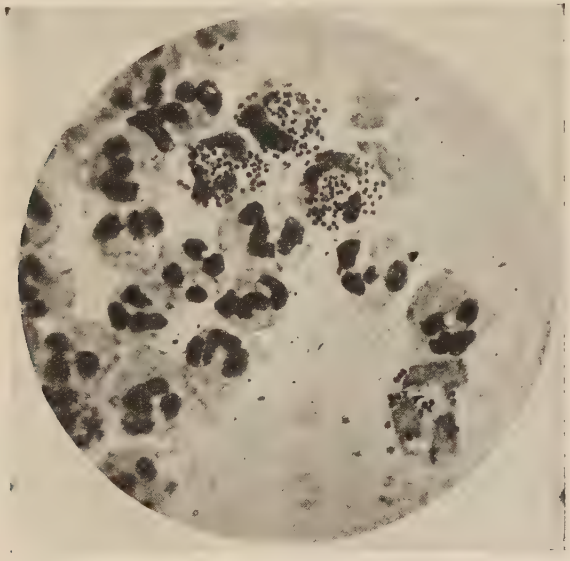


FIG. 259.—Gonococci in urethral pus (× 1000).

There is likewise difficulty in finding gonococci in vaginal discharges unless comparatively pure pus from the suspected lesion can be obtained; otherwise the organisms sought are to a great extent lost among the myriads of bacteria and the epithelial and pus-cells of the leukorrheal discharge. Also, it should be borne in mind that the female genitals frequently harbor a non-pathogenic Gram-negative diplococcus which closely resembles the gonococcus.

PERITONEAL, PLEURAL, AND PERICARDIAL FLUIDS

The serous cavities contain very little fluid normally, but considerable quantities are frequently present as a result of pathologic

conditions. The pathologic fluids are classed as transudates and exudates.

Transudates are non-inflammatory in origin. Their color is light yellow or greenish yellow, and they may be clear, slightly cloudy, or opalescent. They contain only a few cells, and less than 2.5 per cent. of albumin, and do not coagulate spontaneously. The specific gravity is below 1.018. Microörganisms are seldom present.

Exudates are of inflammatory origin. They are richer in cells and albumin, and tend to coagulate upon standing. The specific gravity is above 1.018. The amount of albumin is estimated by Esbach's method, after diluting the fluid, if much albumin be present. A mucin-like substance, called serosomucin, is likewise found in exudates. It is detected by acidifying with a few drops of 5 per cent. acetic acid, when a white cloudy precipitate results. This reaction is very helpful in distinguishing between transudates and exudates, although some transudates give a slight turbidity with acetic acid. Bacteria are generally present and often numerous. When none are found in stained smears of cultures, tuberculosis is to be suspected, and animal inoculation should be resorted to.

Exudates are usually classed as serous, serofibrinous, seropurulent, purulent, putrid, and hemorrhagic, which terms require no explanation. In addition, chylous and chyloid exudates are occasionally met, particularly in the peritoneal cavity. In the chylous form the milkiness is due mainly to the presence of minute fat-droplets, and is the result of rupture of a lymph-vessel, usually from obstruction of the thoracic duct. Chyloid exudates are milky, chiefly from proteins in suspension or fine débris from broken-down cells. These exudates are most frequently seen in carcinoma and tuberculosis of the peritoneum. Blankenhorn believes that all milky effusions are, in reality, chylous, although in some (chyloid, pseudochylous) the fat is so finely divided as to take on some of the properties of colloids.

Cytodiagnosis.—This is diagnosis from a differential count of the cells in a transudate or exudate, particularly one of pleural or peritoneal origin.

A tube of the fresh fluid, obtained by aspiration and, preferably, mixed at once with a little citrated salt solution to prevent clotting, is centrifugalized for at least five minutes; the super-

natant liquid is poured off, and smears are made from the sediment and dried in the air. The fluid must be very fresh, and the smears must be thin and quickly dried, otherwise the cells will be small and shrunken and hence difficult to identify. The smears are then stained with Wright's blood-stain, which has preferably been previously diluted with one-third its volume of pure methyl alcohol. They are examined with an oil-immersion objective.

Predominance of *polymorphonuclear leukocytes* (pus-corpuscles) points to an acute infectious process (Fig. 260). These cells are the neutrophils of the blood. Eosinophils and mast-cells are rare. In thin smears they are easily recognized, the cytoplasmic granules often staining characteristically with polychrome-methylene-blue-

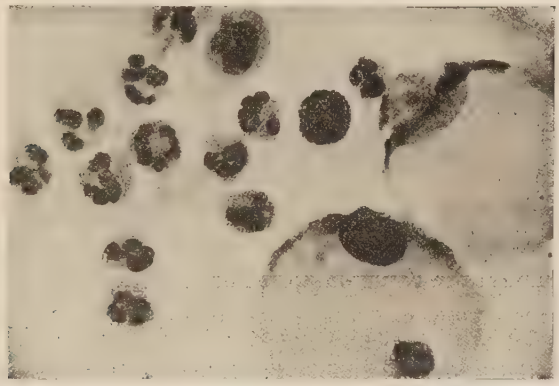


Fig. 260.—Cytodiagnosis. Polymorphonuclear leukocytes and swollen endothelial cells from acute infectious non-tuberculous pleuritis (Percy Musgrave; photo by L. S. Brown).

eosin stains. In thick smears, upon the other hand, they are often small and shrunken, and may be identified with difficulty, being easily mistaken for lymphocytes.

A large number, or even a preponderance, of *eosinophils* is seen in about 1 to 5 per cent. of pleural effusions. The significance is uncertain. Some of these effusions have followed artificial pneumothorax, some others have been of tuberculous origin.

Predominance of *lymphocytes* (Fig. 261) generally signifies tuberculosis. They are the same as found in the blood. The cytoplasm is usually scanty, is often ragged, and sometimes is apparently absent entirely. Tuberculous pleurisy due to direct extension from the lung may give excess of neutrophils owing to mixed infection.

Predominance of *mesothelial cells*, few cells of any kind being present, indicates a transudate (Fig. 262). These cells are large,

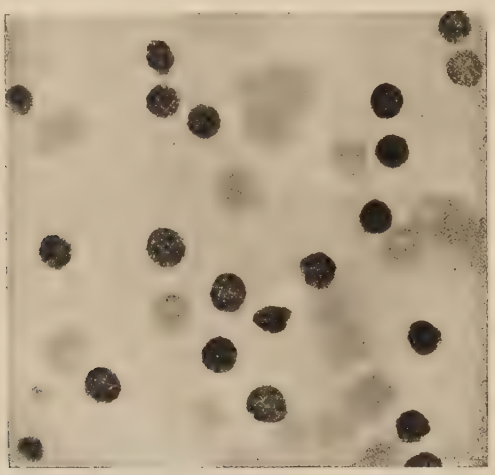


FIG. 261.—Cytodiagnosis. Lymphoid cells from pleural fluid; case of tuberculous pleuritis (Percy Musgrave; photo by L. S. Brown).

with relatively abundant cytoplasm, and contain one, sometimes two, round or oval, palely staining nuclei. Mesothelial cells generally predominate in carcinoma, but are accompanied by consider-

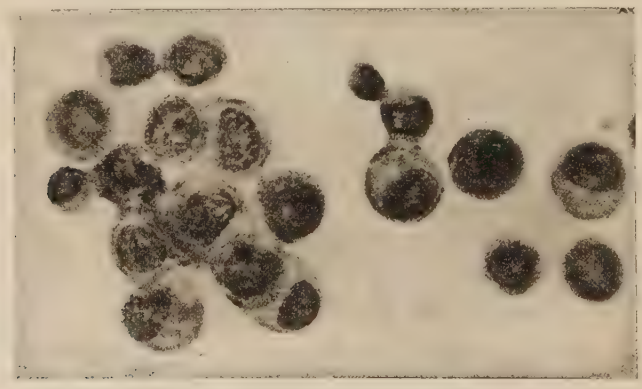


FIG. 262.—Cytodiagnosis. Mesothelial cells from transudate or mechanical effusion (Percy Musgrave; photo by L. S. Brown).

able numbers of lymphocytes and red blood-corpuscles. Cancer cells cannot be recognized as such, although the presence of mitotic figures would suggest malignant disease.

CEREBROSPINAL FLUID

Cerebrospinal fluid is obtained by lumbar puncture between the third and fourth lumbar vertebræ with a flexible needle having a length of about 10 cm. and a bore of from 1 to 1.5 mm., and provided with a stylet. For children a shorter needle is used. The needle and stylet are sterilized by boiling, and the site of the puncture is prepared as described for venous puncture. A local anesthetic is usually used, the skin and subcutaneous tissue being infiltrated with sterile 1 per cent. solution of eucain or novocain. The patient sits upon a stool and bends forward as much as possible, or lies upon his left side at the edge of a bed with knees drawn up and back bent. The site for the puncture is found by running the fingers along the spinous processes of the vertebræ until the "soft spot" between the third and fourth lumbar spines is found. This lies on or just above a line joining the crests of the ilia. The needle is inserted in the midline and pushed directly forward with a quick thrust until it reaches the tough spinous ligaments, when it may be pushed more slowly. A sudden cessation of resistance indicates that it has entered the spinal canal. The stylet is then removed, and the fluid should flow at once. Should it fail to do so, it may usually be started by inserting the needle a little farther or pulling it out a trifle, or by inserting the stylet to dislodge anything that may have clogged the needle, or by having the patient take a deep breath. The pressure under which the fluid appears should be noted. Normally it drips from the needle and the number of drops a minute may be counted. When under high pressure it spurts out. The best method for estimating and recording the pressure is with an Ayer manometer[1] (Fig. 263). Response to pressure on the jugulars is noted at once with this apparatus. In this way a "block" in the spinal canal can be detected.

The fluid is best collected in three sterile test-tubes. One tube receives the first drops, which are usually blood-tinged and unfit for examination. The second tube receives the greater part of the fluid withdrawn. The third should contain a trace of powdered potassium oxalate to prevent clotting. This portion of the fluid is reserved for the cytologic examination. Ordinarily not over 6 to 8 c.c. of fluid should be withdrawn at one time. It is best for the patient to remain in bed for some hours after the puncture.

[1] To be obtained from Codman and Shurtleff, Incorporated, Boston.

Examination of the fluid obtained by lumbar puncture has of recent years become a very important aid in diagnosis, particularly in syphilitic conditions of the nervous system. The routine examination in these cases includes: 1, Macroscopic examination; 2, chemical test for globulin; 3, colloidal gold or mastic test; 4, cytology: (*a*) total cell-count, (*b*) differential cell-count; 5, Wassermann test. When acute infections are in question, the turbidity, bacteriology, and cytology are of chief importance.

1. Macroscopic Examination.—The amount obtainable varies from a few drops to 100 c.c. Normally the fluid is clear and limpid, resembling water. The reaction is alkaline. The specific gravity is 1.003 to 1.008. Not infrequently it is tinged with fresh

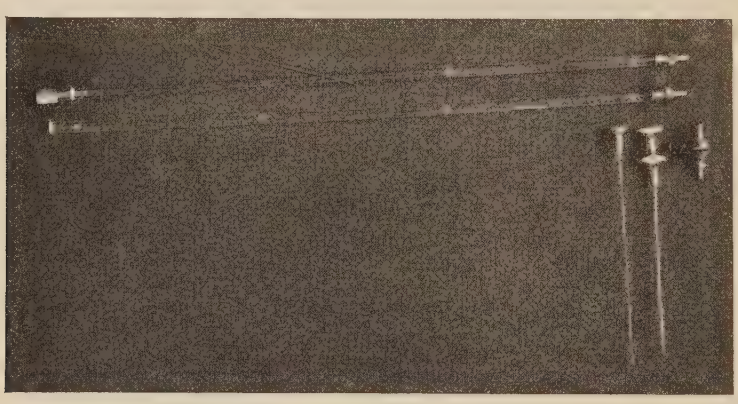


FIG. 263.—Ayer manometer. One part may be fitted into the other, thus making a water manometer either 30 or 60 cm. long. Stop-cock and spinal puncture needle are also shown.

blood from a punctured vessel. This should not be confused with the dull red or brown color which is seen in hemorrhagic conditions, like intraventricular and subdural hemorrhage and hemorrhagic meningitis. When the bleeding is extensive and recent it may give the appearance of practically pure blood.

A condition in which the spinal fluid exhibits a clear yellow color (xanthochromia), usually with rapid and marked coagulation, large amounts of globulin, and no blood-pigments by chemical tests (Froin's syndrome) has been described. It is generally the result of compression by a tumor of the cord or vertebræ with interruption to the flow of cerebrospinal fluid and formation of a pocket of greater or less extent in which the fluid stagnates.

In acute meningitis the fluid may exhibit varying degrees of

cloudiness, from slight turbidity to almost pure pus. In the less acute stage of the epidemic form it is sometimes quite clear. It is also clear in tuberculous meningitis.

After standing for twelve to twenty-four hours the fluid will often coagulate. This occurs especially in the various forms of meningitis, rarely in non-inflammatory conditions. In tuberculosis the coagulum is usually very delicate and cobweb-like, and is not easily seen.

2. Chemical Examination.—Only a few constituents are of clinical importance.

(1) **Proteins.**—Traces are present normally. A notable increase occurs in acute inflammations, in tuberculous meningitis, and in syphilitic and parasyphilitic affections. The chief protein is globulin, and this is the only one considered in clinical work. The three tests for globulin which follow are positive in 93 to 95 per cent. of all cases of paresis, and are, therefore, an important diagnostic consideration. When acute inflammation and tuberculous meningitis are excluded, they run practically parallel with the Wassermann reaction when the latter is applied to the spinal fluid. There may, however, be slight increase of globulin in lethargic encephalitis, poliomyelitis, and other conditions. The globulin test is valueless when applied to fluid containing blood, owing to the presence of serum-globulin.

Noguchi's Butyric Acid Test.—In a small test-tube take 1 to 2 c.c. of the fluid and 5 c.c. of a 10 per cent. solution of butyric acid in normal salt solution. The original test calls for one-tenth these quantities, but they are too small for convenient manipulation. Heat to boiling, and immediately add 1 c.c. of normal sodium hydroxid solution, and boil again for a few seconds. A positive reaction, corresponding to a pathologic amount of globulin, varies from a distinct cloudiness to a heavy flocculent precipitate which generally appears within twenty minutes, but may be delayed for two hours. A slight opalescence may be seen in normal fluids.

Ammonium Sulphate Test.—Globulin is precipitated by strong solutions of ammonium sulphate. Ross and Jones apply the test after the manner of the ring tests for albumin in the urine. In a test-tube or horismascope take a few cubic centimeters of a completely saturated solution of ammonium sulphate, and overlay with the suspected fluid. In the presence of an excess of globulin, a clear-cut, thin, grayish-white

ring appears at the zone of contact of the two fluids within a few seconds. Under normal conditions a ring may appear within five minutes to three hours. This test appears to be fully as reliable as the butyric acid test.

Pandy's test is said to be more definite and more sensitive. The reagent consists of a saturated aqueous solution of phenol crystals, made by adding 10 gm. of the crystals to about 100 c.c. of water, and keeping in an incubator with frequent shaking for several days. To 1 c.c. of the reagent add 1 drop of the cerebrospinal fluid. A bluish-white cloud indicates an abnormal amount of globulin.

Quantitative Method for Protein.—This is seldom used, since the qualitative tests for globulin usually give all the information that is desired.

The *Sicard-Cantelouble method* employs a glass tube of 7 mm. inside diameter, 19 cm. long, graduated in cubic centimeters to 4 c.c., the lower 2 c.c. being further graduated in 0.2 c.c. The method is as follows:

Place 4 c.c. spinal fluid in the tube, heat to 60 to 80° C., and add 12 drops of $33\frac{1}{3}$ per cent. trichloracetic acid. After five minutes invert a few times. Let stand twenty-four hours, and read quantity of sediment Precipitation to:

First graduation indicates 0.22 gm. protein for each liter.
Second graduation indicates 0.40 gm. protein for each liter.
Third graduation indicates 0.56 gm. protein for each liter.
Fourth graduation indicates 71 gm. protein for each liter.
Fifth graduation indicates 0.85 gm. protein for each liter.

The normal does not exceed 0.30 gm. for each liter.

(2) **Colloidal Gold Test.**—Lange's colloidal gold test, introduced in 1912 and now very widely used, consists in mixing cerebrospinal fluid in certain proportions with a colloidal solution of gold. Normal cerebrospinal fluid causes no change in color. Fluids from cases of syphilis and certain pathologic conditions of the nervous system induce changes in the color of the gold solution from red to purple, deep blue, pale blue, or colorless. Moreover, the dilution at which the maximum color change occurs is more or less characteristic of the different pathologic conditions. The typical "curves" are shown in Figure 264. The test gives its most consistent and valuable results in cases of general paresis. In lethargic encephalitis and poliomyelitis atypical curves in the tabetic zone have been observed.

The exact explanation of the test is not yet wholly clear, but it is undoubtedly dependent upon the presence of a globulin.

The test itself is relatively simple, and any difficulty may be attributed to imperfectly cleaned utensils or to a faulty reagent, the preparation of which is time-consuming and uncertain. The reagent can be purchased ready prepared.

Preparation of Reagent.—Lange's Method (*Modified by Miller, Brush, Hammers, and Felton*).—It is imperative that all water used be triply distilled with avoidance of rubber connections in the still, that the beaker used for heating the solution be of Jena or Nonsol glass, and that all glassware be absolutely clean. It is recommended that the glass be boiled in a solution of Ivory soap, brushed thoroughly under the tap, rinsed well, soaked for one-half hour or longer in hot bichromate cleaning

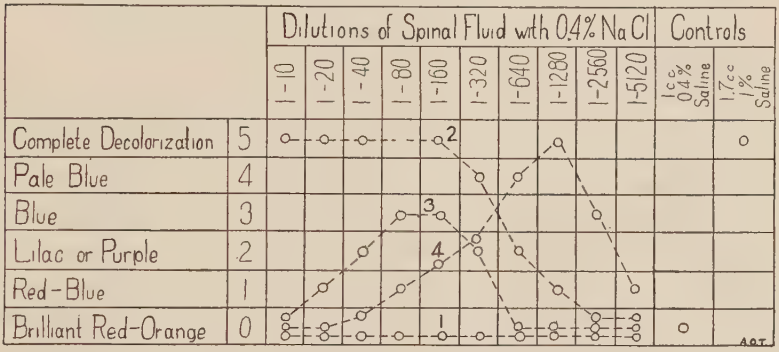


FIG. 264.—Types of reactions in colloidal gold test: 1, Normal cerebrospinal fluid, no reaction; 2, paretic type; 3, luetic or tabetic type; 4, meningitic type.

fluid (p. 644), and immediately before use rinsed thoroughly with distilled water and finally with triply distilled water.

Heat slowly 1000 c.c. triply distilled water in a beaker. When the temperature reaches 60° C. add 10 c.c. of a 1 per cent. solution of chlorid of gold (Merck's yellow crystals in sealed ampules) and 7 c.c. of a fresh 2 per cent. solution of potassium carbonate (Merck's "Blue Label"), using a clean thermometer as a stirring rod. At 80° C. slowly add 10 drops of a 1 per cent. solution of oxalic acid (Merck's "Blue Label"), stirring briskly meanwhile. At 90° C. turn out the fire and add 5 c.c. of a formaldehyd solution containing 1 c.c. of Merck's formaldehyd, "40 per cent., highest purity" in 40 c.c. of distilled water. This must be added drop by drop under constant stirring. Should a pink color develop before all the formaldehyd is added, stop at once, for this will slowly deepen to the brilliant orange-red of the finished solution.

Before use the solution must be neutralized with fiftieth normal hydrochloric acid or fiftieth normal sodium hydroxid, as the case may be. The amount to be added is found by titrating a small portion removed for the purpose, using a 1 per cent. solution of alizarin red in 50 per cent. alcohol as indicator. With an acid reaction this indicator gives a lemon-yellow color; with neutral reaction, brownish red; with alkaline, reddish purple. The solution is worthless unless it meets the following requirements: it must be absolutely transparent and of a brilliant salmon or orange-red color with no trace of blue; it must be neutral to alizarin-red on the day it is used; a 5 c.c. sample must be completely decolorized in one hour when added to 1.7 c.c. of 1 per cent. sodium chlorid; it must give a typical paretic curve with a known paretic spinal fluid, and must not give a reaction beyond "red-blue" with a normal spinal fluid.

Technic of Test.—Arrange a series of twelve clean test-tubes in a rack. Place 1.8 c.c. fresh sterile 0.4 per cent. solution of sodium chlorid in the first test-tube and 1 c.c. in each of the others except the twelfth. In the twelfth tube place 1.7 c.c. of sterile 1 per cent. sodium chlorid. To the first tube add 0.2 c.c. of the spinal fluid, which must be free from any trace of blood. Mix well by sucking the fluid up into the pipet and expelling it, and then transfer 1 c.c. to the second tube. Mix and transfer 1 c.c. to the third tube, repeating this down the row to the tenth tube and discarding the last 1-c.c. portion. This leaves the eleventh and twelfth tubes with salt solution only to serve as controls. To each of these twelve tubes add 5 c.c. of the colloidal gold solution. Let stand at room temperature for an hour or longer, at the end of which time, in the case of a positive reaction, the solution in some of the tubes will have changed from red to purple, deep blue, pale blue, or colorless. In the case of normal fluids no change will occur. The fluid in the eleventh and twelfth tubes which serve as controls should be orange-red and colorless, respectively. The results are usually charted, as shown in Figure 264, in which each column represents a tube. For the purpose of brevity the colors may be indicated by the corresponding numbers, which are placed in the same order as the tubes. Thus the "paretic reaction" in Figure 264 may be expressed as 5555542100. Felton's suggestion that the type of reaction is best indicated by using the terms Zone I, Zone II, Zone III is used in many laboratories for reporting the "paretic," "tabetic," or "meningitic" curves.

(3) **Mastic Test.**—Because of the many difficulties in the way of preparing satisfactory and uniform colloidal gold solutions, the mastic test has been proposed as a substitute for the gold test. The

reagent is inexpensive and easily made, and the test is easily carried out. Results appear to parallel those obtained with colloidal gold, being almost uniformly positive in paresis, cerebrospinal syphilis, and tabes; but there is much less definite differentiation of the various types of reaction Complete precipitation of the mastic corresponds to complete decolorization of the colloidal gold solution; while partial precipitation of the mastic corresponds roughly to the purple or blue of the colloidal gold test. The method which follows is that used by Cutting:

Preparation of Solutions.—(*a*) *Mastic Solution.*—Make a stock solution by completely dissolving 10 gm. of gum mastic, U. S. P., in 100 c.c. of absolute alcohol and filter. To 2 c.c. of this stock solution add 18 c.c. of absolute alcohol, mix well, and pour rapidly into 80 c.c. of distilled water.

(*b*) *Alkaline-saline Solution.*—Make a 1.25 per cent. solution of sodium chlorid (C. P.) in distilled water, and to each 99 c.c. of this solution add 1 c.c. of a 0.5 per cent. solution of potassium carbonate in distilled water.

Technic of Test.—Arrange a series of six small test-tubes. In the first place 1.5 c.c. of the alkaline-saline solution and in each of the others place 1 c.c. To the first tube add 0.5 c.c. of the spinal fluid, which must be completely free from blood. Mix by sucking the fluid up into the pipet and expelling it, and transfer 1 c.c. to the second tube. Again, mix and transfer 1 c.c. to the third tube and continue down the line to the fifth tube, discarding the 1-c.c. portion which is removed from this, and leaving the sixth tube with alkaline saline solution alone to serve as a control. Finally add 1 c.c. of the mastic solution to each tube. Mix well and set aside at room temperature for twelve to twenty-four hours, or in the incubator for six to twelve hours. Tubes in which the reaction is complete will show a heavy precipitate with clear supernatant fluid (Fig. 265).

(4) **Colloidal Benzoin Test.**—This test, devised by Guillain, Laroche, and Lechelle, is similar in many respects to the mastic test. It is not specific for neurosyphilis, but does give practically the same results as the more complicated colloidal gold test.

Reagents.—(*a*) *Benzoin Solution.*—Sumatra benzoin resin, 1 gm.; absolute alcohol, 10 c.c. After forty-eight hours filter off the clear supernantant fluid. Keep in a tightly stoppered bottle. This is a stock

solution from which the colloidal solution which is used in the test is freshly prepared each day as follows:

Add 0.3 c.c. of the stock benzoin solution, drop by drop with constant shaking, to 20 c.c. of doubly distilled water. Heat to 35° C. in a water-bath with constant shaking.

(*b*) *Salt Solution.*—Make 0.01 per cent. sodium chlorid in doubly distilled water.

Technic of Test.—Set up in a rack sixteen small test-tubes (75 by 10 mm., or 85 by 13 mm.). In the first tube place 0.25 c.c. of salt solution (*b*); in the second tube 0.5 c.c.; in the third 1.5 c.c., and in each of the remaining tubes 1 c.c. Next add cerebrospinal fluid: 0.75 c.c. to the first tube; 0.5 c.c. to the second and third tubes. From tube three 1 c.c. of the thoroughly mixed dilution of spinal fluid is transferred

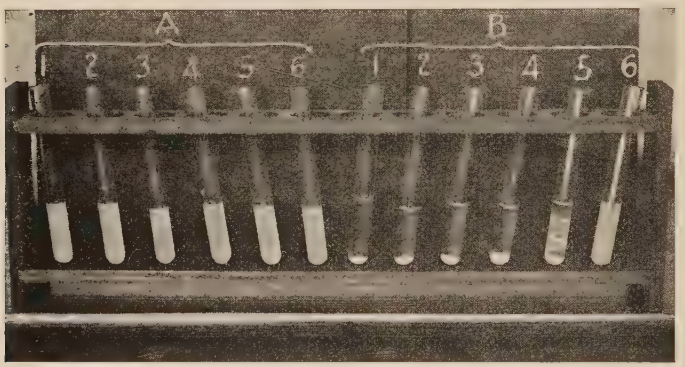


FIG. 265.—The mastic reaction in cerebrospinal fluid: *A*, From a case of dementia præcox, negative; *B*, from a case of paresis, positive. (Courtesy of Jas. A. Cutting.)

to the fourth tube, and so on, until the fifteenth tube is reached, from which, after mixing, 1 c.c. is discarded. The sixteenth tube is used for control. The dilutions thus range from 3 parts in 4 in the first tube to 1 in 16,384 in the fifteenth tube. Finally, add 1 c.c. of the benzoin suspension (*a*) to each tube, and mix by shaking. The tubes are allowed to stand for from eighteen to twenty-four hours.

The reaction will vary from no change in the mixture to complete precipitation of the benzoin, with absolute clearing of the supernatant fluid. The degree of reaction in each tube is reported: 0, no precipitation; 1, slight precipitation, with partial clearing; 2, more than half precipitated, fluid still cloudy; 3, complete precipitation, water-clear fluid. A curve may be plotted, or the figures representing the degree of reaction may be set down for each tube. Precipitation in the first six tubes indicates cerebral involvement, the first, or paretic zone; precipita-

tion beginning with the seventh tube indicates involvement of the meninges, or spinal cord, the second, or meningeal zone. The test is not as sensitive as in the Lange colloidal gold method, and is not so definite in its reaction in multiple sclerosis.

(5) **Sugar.**—The normal cerebrospinal fluid contains about 0.04 to 0.07 per cent. of dextrose, or roughly, about one-half as much as the blood. This is sufficient to give a distinct reaction with Benedict's test (p. 147), but it is well to use twice as much of the cerebrospinal fluid as is recommended for the urine. This reduction of copper is diminished or absent in certain forms of meningitis, probably owing to destruction of dextrose by the bacteria. From a study of a series of cases Jacob finds that: (1) No reduction of copper occurs in pyogenic meningitis (pneumococcus, streptococcus) or in acute meningococcic meningitis; (2) reduction occurs, but may be diminished in tuberculosis and in the more chronic cases of meningococcic meningitis; (3) reduction is normal in poliomyelitis. Increased reduction occurs in diabetes mellitus, and a moderate increase is sometimes observed in lethargic encephalitis.

Quantitative estimation, which is rarely undertaken, may be carried out by the methods already given for blood-sugar. For the method of Folin and Wu (p. 348) dilute 1 volume of spinal fluid with 4 volumes of water and use in place of the protein-free blood-filtrate. For the method of Lewis and Benedict use 4 c.c. of spinal fluid and 6 c.c. of water in step 1, page 350. There must, of course, be a corresponding change in the calculation.

(6) **Antimeningococcus-serum Test.**—Vincent and other French investigators have developed the following test, which they believe to be specific for epidemic cerebrospinal meningitis:

To 1 c.c. of the spinal fluid, which has been cleared by thorough centrifugation, are added a few drops of antimeningococcus serum. The tube, along with a control tube of the untreated fluid, is then placed in an incubator at 52° C. for a few hours. A positive reaction consists in the appearance of a white cloud. The present view is that the test has some value, but cannot be wholly relied upon. Sometimes a positive reaction occurs in other forms of meningitis. Sometimes both tubes become cloudy during the incubation.

3. Microscopic Examination.—This consists in a study of the bacteria, and of the number and kind of cells.

(1) **Bacteria.**—*Tubercle bacilli* can be found in the great majority of cases of tuberculous meningitis. The delicate coagulum which forms when the fluid is allowed to stand in a cool place for twelve to twenty-four hours will entangle any bacilli which may be present. This clot may be removed, spread upon slides, and stained by one of the methods already given (pp. 65–67). Still better is a method proposed by Sheo-Nan Cheer: To a portion of the spinal fluid in a centrifuge tube add with constant, gentle shaking, one-third to one-half its volume of 95 per cent. alcohol and centrifugalize thoroughly at high speed. Just enough alcohol should be added to precipitate a slight cloud of proteins which

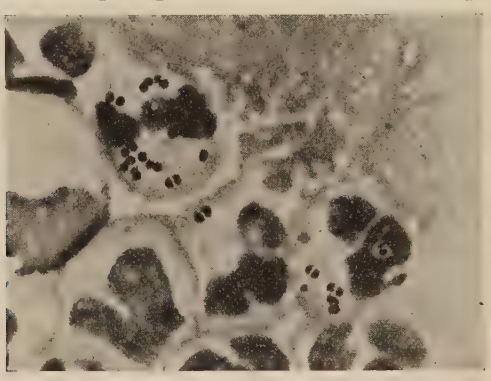


Fig. 266.—Meningococci in cerebrospinal fluid from a case of epidemic spinal meningitis. Gram's method and carbolfuchsin (photograph × 1500).

will carry the tubercle bacilli to the bottom. If the fluid gives no precipitate, a trace of egg-albumin should be added. It may be necessary to examine a considerable number of smears. In doubtful cases inoculation of guinea-pigs must be resorted to.

The *Diplococcus intracellularis meningitidis* or *meningococcus* is the cause of epidemic cerebrospinal fever, and can be detected in the cerebrospinal fluid of most cases, especially those which run an acute course. Cover-glass smears from the sediment should be stained by a simple bacterial stain and by Gram's method. The meningococcus is an intracellular diplococcus which often cannot be distinguished from the gonococcus in stained smears (Fig. 266). It also decolorizes by Gram's method. The presence of such a diplococcus in meningeal exudates is, however, sufficient for its

identification in clinical work. In acute meningitis the diplococci can sometimes be found within neutrophilic leukocytes in ordinary blood-smears.

When the meningococci are not found in direct smears from a spinal fluid which contains many pus-corpuscles we have had excellent results with Obe's enrichment method. Enough sterile 10 per cent. dextrose solution is added to make about a 1 per cent. solution, and the fluid is placed in the incubator for ten or twelve hours. The number of meningococci very greatly increases; they are taken up by the leukocytes and can be found in the sediment microscopically. Cultural methods are described on page 669.

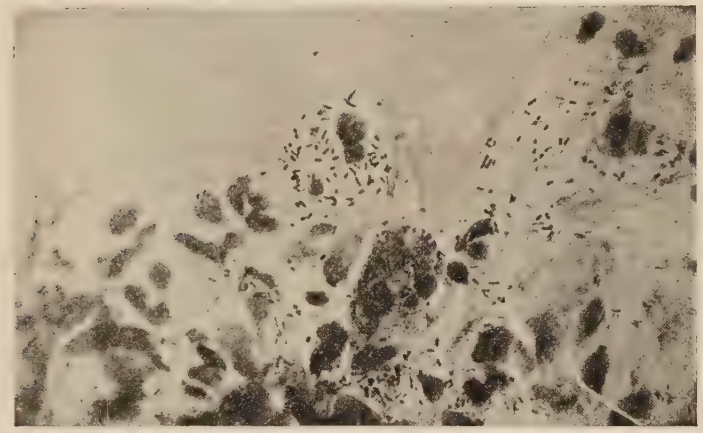


FIG. 267.—Influenza bacilli in spinal fluid. Case of meningitis (photograph × 1000).

The meningococcus is present in the nasal discharge in most cases of this form of meningitis, and in very small numbers in the posterior nares and nasopharynx of some healthy persons who are dangerous as carriers. In this situation they can be identified only by cultures.

Various organisms have been found in other forms of meningitis—the pneumococcus most frequently, the influenza bacillus (Fig. 267) rarely. When the pneumococcus is present it is usually very abundant. In some cases no microörganisms can be detected even by cultural methods.

(2) **Parasites.**—The larvæ of Trichinella have been found in the spinal fluid at the same time that they are present in the blood (p. 320). Trypanosomes are present in the late stages of African sleeping sickness.

(3) **Cytology.**—The fluid should be as fresh as possible, as the cells tend to degenerate. To avoid formation of a coagulum which might entangle the cells and interfere with the count it is well to secure the fluid in two tubes, one of which, used for the cytologic examination, contains a trace of powdered potassium oxalate. Early coagulation is, however, not common in the diseases in which the cell count is most important.

The routine examination should include both a total and a differential count

The **total cell count** is best made with the Fuchs-Rosenthal counting chamber in a manner similar to the counting of leukocytes in blood. The ruled area (Fig. 268) in this chamber covers 16 sq. mm. and the depth below the cover-glass is 0.2 mm. The capacity is thus a trifle more than 3 cu. mm. (sixteen-fifths). Unna's polychrome methylene-blue or a staining fluid consisting of crystal-violet 0.1 gm., glacial acetic acid 1.0 c.c., water 50 c.c., 5 per cent. phenol a few drops, is drawn into the leukocyte pipet to the mark 1, and the fresh spinal fluid, which has been well shaken, is drawn up to the mark 11. After mixing, a drop is placed in the counting chamber and covered. The number of cells in the entire ruled area is counted and this number divided by 3 to give the number of cells for each cubic millimeter of spinal fluid. The error incident to this calculation is practically balanced by the opposite error due to the dilution.

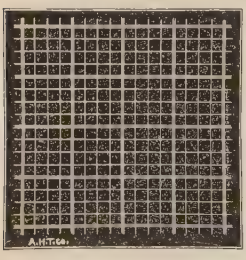


FIG. 268.—Fuchs-Rosenthal ruling of counting-chamber for cells in spinal fluid.

When the Fuchs-Rosenthal chamber is not at hand the ordinary hemacytometer counting chamber may be substituted. The number of cells in the ruled area is counted, the number for each cubic millimeter is calculated, and this is multipled by $\frac{10}{9}$ to compensate for the dilution with staining fluid.

The **differential count** is made as described on page 284. A weak aqueous solution of methylene-blue is probably preferable to Wright's stain for this purpose. Ordinarily, only two kinds of cells are seen: lymphocytes and polymorphonuclear neutrophils.

The cells normally present are nearly all lymphocytes. They vary in number from 1 to 5 or 7 in each cubic millimeter; 10 is usually accepted as the maximum in health.

An increase in the cell count, together with predominance of lymphocytes (more than 70 per cent.), strongly suggests tuberculous meningitis or syphilitic disease of the nervous system since it occurs in about 90 to 95 per cent. of the cases. The number of cells present in these conditions varies greatly in different cases, but ordinarily lies between 25 and 100 in each cubic millimeter. Similar counts are frequent in lethargic encephalitis and anterior poliomyelitis, and may also sometimes be noted in cerebral hemorrhage, tumors, and the more chronic type of epidemic cerebrospinal meningitis.

In all forms of acute meningitis the total count is high, 100 to several thousand, and polymorphonuclear leukocytes prevail. A notable number of endothelial cells may also be present, especially in acute epidemic meningitis.

ANIMAL INOCULATION

Inoculation of animals is one of the most reliable means of verifying the presence of certain micro-organisms in fluids and other pathologic material, and is helpful in determining the species of bacteria which have been isolated in pure culture.

Clinically, it is applied most frequently to demonstration of the tubercle bacillus when other means have failed or are uncertain. The guinea-pig is the most suitable animal for this purpose. When the suspected material is fluid and contains pus, it should be well centrifugalized, and 1 or 2 c.c. of the sediment injected, by means of a large hypodermic needle, into the peritoneal cavity or underneath the loose skin of the groin. Fluids from which no sediment can be obtained must be injected directly into the peritoneal cavity, since at least 10 c.c. are required, which is too great an amount to inject hypodermically, and several animals should be inoculated, since some are likely to die from peritonitis caused by other organisms before the tubercle bacillus has had time to produce its characteristic lesions. For intraperitoneal injection the animal should be held with its head downward to cause the intestines to gravitate toward the diaphragm. The needle is then introduced between the umbilicus and the pelvis. Solid material should be placed in a pocket made by snipping the skin of the groin with scissors, and freeing it from the underlying tissues for a short distance around the opening.

The animals should be killed at the end of six or eight weeks, if

they do not die before that time; and a careful search should be made for the characteristic pearl-gray or yellow tubercles scattered over the peritoneum and through the abdominal organs, particularly the spleen and liver, and for caseous inguinal and retroperitoneal lymph-glands. The tubercles and portions of the caseous glands should be crushed between two slides, dried, and stained for tubercle bacilli. The bacilli are not usually abundant in the caseous material and may, therefore, be difficult to find.

CHAPTER VIII

MISCELLANEOUS EXAMINATIONS

THE NOSE, MOUTH, AND PHARYNX

Micro-organisms are always present in the mouth in large numbers. Among these is *Leptotrichia buccalis* (Fig. 269), which is especially abundant in the crypts of the tonsils and the tartar of the teeth. The whitish patches of *pharyngomycosis leptothrica* are largely composed of these fungi. They are slender, segmented

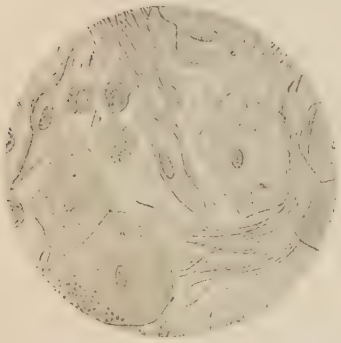


Fig. 269.—Gingival deposit (unstained): *a*, Squamous epithelial cells; *b*, leukocytes; *c*, bacteria; *d*, *Leptotrichia buccalis* (Jakob).

Fig. 270.—Thrush fungus (*Endomyces albicans*) (Kolle and Wassermann).

threads, which generally, but not always, stain violet with Lugol's solution, and are readily seen with a 4-mm. objective. Often the segments are separated, and then have the appearance of very large non-motile bacilli. At times they are observed in the sputum and stomach fluid. In the former they might be mistaken for elastic fibers; in the latter, for Boas-Oppler bacilli. In either case the reaction with iodin will distinguish them.

The prevalence of endamebæ and spirochetes in the mouths of normal persons and of those suffering from pyorrhea alveolaris has already been mentioned (pp. 453 and 462).

Thrush is a disease of the mouth seen most often in children, and characterized by the presence of white patches upon the mucous

membrane. It is caused by the thrush fungus, *Endomyces* (*Monilia*) *albicans*. When a bit from one of the patches is pressed out between a slide and cover and examined with a 4-mm. objective, the fungus is seen to consist of a network of simple or branching segmented filaments (Fig. 270). The segments measure about 4 by 20 μ. Globular or oval cells of somewhat greater diameter are attached to the filaments or lie free in the meshes between them. The meshes also contain leukocytes, epithelial cells, and granular débris. A related fungus, *Monilia psilosis*, is found in scrapings from the tongue lesions of sprue.

Acute pseudomembranous inflammations, which occur chiefly upon the tonsils and nasopharynx, are generally caused by

FIG. 271.—*Bacillus diphtheriæ* stained with methyl-green; culture from throat (photograph × 1000).

the diphtheria bacillus, but may result from streptococcic infection. In many cases diphtheria bacilli can be demonstrated in smears made from the membrane and stained with Löffler's methylene-blue or 2 per cent. aqueous solution of methyl-green or, much better, Albert's stain, given below. It is generally necessary, however, and always safer, to make a culture from the throat swabs upon Löffler's blood-serum, and to examine smears from the growth at the end of eight, twelve, twenty-four, and thirty-six hours. The bacilli are straight or curved rods, which vary markedly in size and outline and stain very irregularly. A characteristic form is a palely tinted rod with several deeply stained granules

(metachromatic granules), or with one such granule at each end (Fig. 271). Sometimes they are club shaped or barred. There is a strong tendency to parallelism—for several to lie side by side. They are Gram-positive. In clinical diphtheria the bacilli generally dominate every field, provided the swab was properly made. In "carriers" often only occasional small groups can be found in the film.

Sometimes bacilli are found which are morphologically typical, but avirulent. These may be recognized by isolating them and inoculating a guinea-pig subcutaneously or intraperitoneally with 1 c.c. of a forty-eight- to seventy-two-hour broth culture. If the strain be virulent, the animals should die within two days. As a control it is well to inject into a second guinea-pig the same amount of culture to which have been added 100 to 200 units of diphtheria antitoxin. This pig should not die.

Neisser's stain has long been the standard differential stain for the diphtheria bacillus. It colors the bodies of the bacilli brown and the metachromatic bodies blue.

1. Make films and fix as usual.
2. Apply the following solution, freshly filtered, for about one-half minute:

Methylene-blue	0.1 gm.
Alcohol (96 per cent.)	2.0 c.c.
Glacial acetic acid	5.0 "
Distilled water	95.0 "

3. Rinse in water.
4. Apply a saturated aqueous solution of Bismarck brown one-half minute.
5. Rinse, dry, and mount.

Albert's Method.—We have found this more satisfactory than Neisser's.

1. Prepare films, dry, and fix in the flame.
2. Apply the following staining solution for one minute:

Toluidin blue	0.15 gm.
Methyl green	0.2 "
Glacial acetic acid	1.0 c.c.
Alcohol (95 per cent.)	2.0 "
Distilled water	100.0 "

Mix well, let stand twenty-four hours, and filter.

3. Rinse with water and blot dry.
4. Apply the following iodin solution for one minute:

Iodin	2.0 gm.
Potassium iodid	3.0 "
Distilled water	300.0 c.c.

5. Rinse in water, dry, and examine.

Diphtheria bacilli are green, bars dark green, granules black and very prominent. Nearly all other organisms take a light green stain. The formula as published has proved very satisfactory; however, some workers prefer to substitute malachite-green for methyl-green.

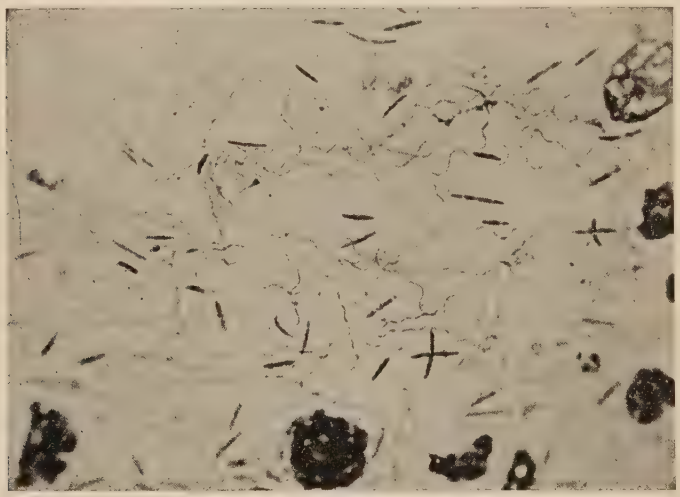


FIG. 272.—*Borrelia vincenti* from case of ulcerative stomatitis stained with gentian-violet (photograph × 1200).

Vincent's angina is a pseudomembranous and ulcerative inflammation of mouth and pharynx, which when acute may be mistaken for diphtheria, and when chronic is very apt to be mistaken for syphilis. Stained smears from the ulcers or membrane show large numbers of spirochetes and fusiform bacilli, giving a striking and characteristic picture (Fig. 272). Before making the smears the surface of the lesion should be gently cleaned by swabbing, otherwise so many saprophytic bacteria may be present that the characteristic picture is obscured. The bacillus is spindle shaped, more or less pointed at the ends, and about 4 to 8 μ long. The spirillum is a very slender, wavy thread, about 10 to 20 μ long,

and stains feebly. Diluted formalin-gentian-violet makes a satisfactory stain. Giemsa's stain is also satisfactory. With methylene-blue the palely staining spirillum may easily be overlooked. Their reaction to Gram's stain is variable. Further description is given on page 460.

Tuberculous ulcerations of mouth and pharynx can generally be diagnosed from curettings made after careful cleansing of the surface. The curettings are well rubbed between slide and cover, and the smears thus made are dried, fixed, and stained for tubercle bacilli. Since there is much danger of contamination from tuberculous sputum, the presence of tubercle bacilli is significant only in proportion to the thoroughness with which the ulcer was cleansed. The diagnosis is certain when the bacilli are found within groups of cells which have not been dissociated in making the smears.

In **leprosy** with intranasal ulcerations smears made from the nasal secretions or, better, from swabs of the ulcers themselves, constitute a useful aid to diagnosis. They often show the leprosy bacilli in great numbers, generally lying within large cells. They are stained as are tubercle bacilli, but with especial care not to decolorize, since these bacilli are not so strongly acid-fast as are tubercle bacilli. Leprosy bacilli can also be found, and are often abundant in material aspirated from any accessible nodular lesion, as is described in a later section for aspiration of *Treponema pallidum* from lymph-nodes.

SALIVA

The secretion of the salivary glands amounts to about 1500 c.c. in twenty-four hours. It is a colorless, odorless, slightly viscid liquid with alkaline reaction. Microscopically it contains a few squamous epithelial cells; a few "salivary corpuscles," which are chiefly mononuclear blood-corpuscles, generally much swollen and often filled with granules in active Brownian motion; and many miscellaneous bacteria, the number depending largely on the condition of the teeth and gums. Most of the microörganisms are saprophytic and many will not grow on ordinary culture-media; but pneumococci, chiefly Types III and IV, streptococci, and other pathogenic organisms are not infrequently present in the saliva of healthy persons.

Physiologically speaking, the most important constituent of

saliva is salivary amylase or ptyalin. Its presence may be recognized by its digestion of starch. To a few cubic centimeters of saliva in a test-tube are added a few drops of a 1 per cent. solution of starch, and the tube is placed in an incubator or water-bath at about 37° C. for ten or fifteen minutes. At intervals a drop of the fluid is removed and mixed with a drop of Gram's iodin solution on a white porcelain plate. If ptyalin be present, the successive drops so treated will turn blue, purple, red, and finally yellow, owing to the gradual transformation of starch into the successive products of indigestion, erythrodextrin and achroödextrin, and finally into maltose, which is capable of reducing Benedict's copper solution.

From the clinical point of view an important constituent of saliva is urea. As is well known, there is a tendency to uniform distribution of urea among the body fluids, and Hench and Aldrich have shown that the combined urea and ammonia nitrogen of the saliva approximates the concentration of urea nitrogen in the blood, and thus furnishes a useful index of renal functional capacity. The sum of the urea and ammonia nitrogen is taken because urea tends to break down into ammonium carbonate through the action of bacteria after the saliva is secreted, and the ammonia of the saliva therefore really represents urea. Saliva is obtained for examination as described below. It is filtered through several layers of gauze, and diluted as described in the footnote on page 338. Urea is then estimated by the method for blood urea (p. 337) which gives urea and ammonia nitrogen together. Normally, saliva contains from 6 to 16 mg. of combined urea and ammonia nitrogen for each 100 c.c. corresponding to about 13 and 36.5 mg. urea; this increases with any increase of urea in the blood, although the rate of increase is somewhat less. The concentration of urea in the saliva is apparently very little influenced by salivation or the action of pilocarpin.

More recently Hench utilized the mercury-combining power of saliva as an approximate method of determining urea and hence as an index of renal insufficiency. The power to bind mercury is due to various nitrogenous substances in saliva, but chiefly to urea. The method is extremely simple and requires not more than five or ten minutes, hence it is available as an office procedure. It cannot take the place of blood-urea determinations. When blood chemical methods are not available, or when venipuncture is impossible or impracticable, it offers a simple means of determining

whether nitrogen retention exists and approximately the degree; and it may be used as a preliminary procedure when resort to blood chemistry is contemplated.

Mercury-combining Power of Saliva.—Method of Hench and Aldrich.[1] *Reagents Required.*[2]—(*a*) Mercuric chlorid solution, an accurately prepared 5 per cent. solution of chemically pure mercuric chlorid in distilled water. (*b*) Sodium carbonate, saturated solution in distilled water.

Collection of Saliva.—The mouth is well rinsed with water. Chewing of a small piece of paraffin or holding a small marble in the mouth will favor the flow of saliva, but this is not necessary. The saliva is collected in two portions of about 8 c.c. each. The first of these carries off food particles and epithelial débris and is discarded. The second is used for the titration. It need not be filtered.

Method.—1. By means of a pipet transfer 5 c.c. of the saliva to a small flask or beaker.

2. Add 5 per cent. solution of mercuric chlorid from a buret or pipet a few drops at a time, with constant stirring, until a drop of the fluid, when added to a drop of saturated solution of sodium carbonate on a white porcelain plate, gives a definite reddish-brown color. The color should appear within about three seconds. If it develops more slowly, the end-point is near, but not yet reached, and a few additional drops of the bichlorid must be added.

3. When the end-point is reached note the number of cubic centimeters of mercuric chlorid solution which have been added, and multiply by 20 to find the number of cubic centimeters which would be required for 100 c.c. of saliva. Record this as the "mercury-combining index."

Hench has found the mercury-combining index in normal persons to lie between 30 and 50 for 100 c.c. of saliva. When there is retention of urea in the blood the index rises with the blood-urea, although it lags a little behind. The probable blood-urea may be roughly calculated as follows:

1.43 × salivary index − 34 = Probable blood-urea in milligrams for each 100 c.c.

Example: Suppose the salivary index were 100. Then 1.43 × 100 − 34 = 109 mg. urea in 100 c.c. of blood.

[1] Hench, P. S., and Aldrich, Martha: A Salivary Index to Renal Function, Jour. Amer. Med. Assoc., vol. 81, p. 1997, December, 1923.

[2] For the convenience of the practitioner the necessary reagents and simple apparatus have been assembled in a small box and put on the market by Hynson, Westcott, and Dunning.

The significance of retention of urea in the blood as an index of kidney insufficiency is discussed on page 104.

THE EYE

For studying the bacteriology of the eye both smears and cultures should be made. The former are much the more useful in many cases. In the normal conjunctiva *Staphylococcus albus*, *Bacillus xerosis*, and Type IV pneumococcus are the bacteria most commonly found.

Staphylococci, pneumococci, and **streptococci** are probably the most common of the bacteria to be found in non-specific conjunctivitis and keratitis. Serpiginous ulcer of the cornea is generally associated with the pneumococcus (Fig. 258).

FIG. 273.—Conjunctival secretion from acute contagious conjunctivitis; polynuclear leukocytes with the bacillus of Weeks: *P*, Phagocyte containing bacillus of Weeks (oil-immersion objective, ocular III) (Morax).

The usual cause of acute infectious conjunctivitis ("pink-eye"), especially in cities, seems to be the **Koch-Weeks bacillus.** This is a minute, slender rod, which lies within and between the pus-corpuscles (Fig. 273), and is negative to Gram's stain. In smears it cannot be distinguished from the influenza bacillus, although its length is somewhat greater.

The **diplobacillus of Morax and Axenfeld** gives rise to an acute or chronic blepharoconjunctivitis without follicles or membrane, for which zinc sulphate seems to be a specific. It is widely distributed geographically and is common in many regions. The organism is a short, thick diplobacillus, is frequently intracellular, and is Gram-negative (Fig. 274). A delicate capsule can sometimes be made out.

Early diagnosis of gonorrheal ophthalmia is extremely important, and can be made with certainty only by detection of **gonococci** in the discharge. They are easily found in smears from untreated cases. After treatment is begun they soon disappear, even though the discharge continues.

Pseudomembranous conjunctivitis generally shows either **streptococci** or **diphtheria bacilli.** In diagnosing diphtheric conjunctivitis one must be on his guard against the **Bacillus xerosis,** which is a frequent inhabitant of the conjunctival sac in healthy

persons, and which is identical morphologically with the diphtheria bacillus. Hence the clinical picture is more significant than the laboratory findings unless cultures be made.

Various micro-organisms—bacteria, molds, protozoa—have been described in connection with trachoma. Certain minute intracellular bodies were thought to be the causative agents. These are best seen in smears stained with Giemsa's stain and appear as minute blue dots usually grouped in clusters in the cytoplasm of epithelial cells. A red-staining granule can be seen in many of the blue bodies. The nature of these **"trachoma bodies"** is not yet settled. Recently Noguchi has succeeded in isolating a small bacillus which is now believed to be the cause of trachoma.

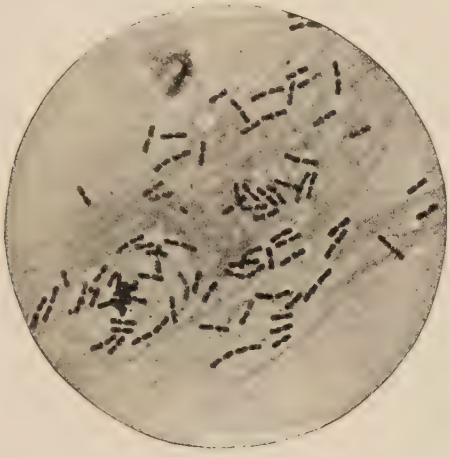


FIG. 274.—The diplobacillus of Morax and Axenfeld (from a preparation by Dr. Harold Gifford).

Herbert has called attention to the abundance of eosinophilic leukocytes in the discharge of **vernal catarrh.** He regards their presence in considerable numbers as very helpful in the diagnosis of this disease.

THE EAR

By far the most frequent exciting causes of acute otitis media are the pneumococcus and the streptococcus. The finding of other bacteria in the discharge generally indicates a secondary infection, except in cases complicating infectious diseases, such as typhoid fever, diphtheria, and influenza. Discharges which have continued for some time are practically always contaminated with the staphylococcus. The presence of the streptococcus should be a

cause of uneasiness, since it much more frequently leads to mastoid disease and meningitis than does the pneumococcus. The staphylococcus, bacillus of Friedländer, colon bacillus, and *Bacillus pyocyaneus* may be met in chronic middle-ear disease.

In tuberculous disease the tubercle bacillus is present in the discharge, but its detection offers some difficulties. It is rarely easy to find, and precautions must always be taken to exclude the smegma and other acid-fast bacilli (p. 69), which are especially liable to be present in the ear. Rather striking is the tendency of old squamous cells to retain the red stain, and fragments of such cells may mislead the unwary.

PARASITIC DISEASES OF THE SKIN

Favus, tinea versicolor, and the various forms of ringworm are caused by members of the fungus group. To demonstrate them, a crust or a hair from the affected area is softened with a few drops of 10 per cent. caustic soda solution, pressed out between a slide and cover, and examined with a 4-mm. objective. They consist of a more or less dense network of hyphæ and numerous round or oval refractive spores. The cuts in standard works upon diseases of the skin will aid in differentiating the members of the group. For accurate study of these fungi it is necessary to resort to cultures for which Sabouraud's medium is well adapted.

MILK

A large number of analyses of human and cows' milk are averaged by Holt as follows, Jersey milk being excluded because of its excessive fat:

	Human Milk Normal variations, per cent.		Cows' Milk Average, per cent.	
Fat	3.00 to	5.00	4.00 to	3.50
Sugar	6.00 to	7.00	7.00 to	4.30
Proteins	1.00 to	2.25	1.50 to	4.00
Salts	0.18 to	0.25	0.20 to	0.70
Water	89.82 to	85.50	87.30 to	87.50
	100.00	100.00	100.00	100.00

The reaction of human milk is slightly alkaline; of cows', neutral or slightly acid. The specific gravity of each is about 1.028 to 1.032. Human milk is sterile when secreted, but derives a few

bacteria from the lacteal ducts. Cows' milk, as usually sold, contains large numbers of bacteria, the best milk rarely containing fewer than 10,000 in each cubic centimeter. Microscopically, human milk is a fairly homogeneous emulsion of fat, and is practically destitute of cellular elements. Any notable number of leukocytes indicates infection of the mammary gland.

Chemical examination of milk is of great value in solving the problems of infant feeding. The sample examined should be the middle milk, or the entire quantity from one breast, or, as recommended by Talbot, 1 ounce obtained before nursing, 1 ounce after nursing, the two being then mixed. The fat and protein can be estimated roughly, but accurately enough for many clinical purposes by means of

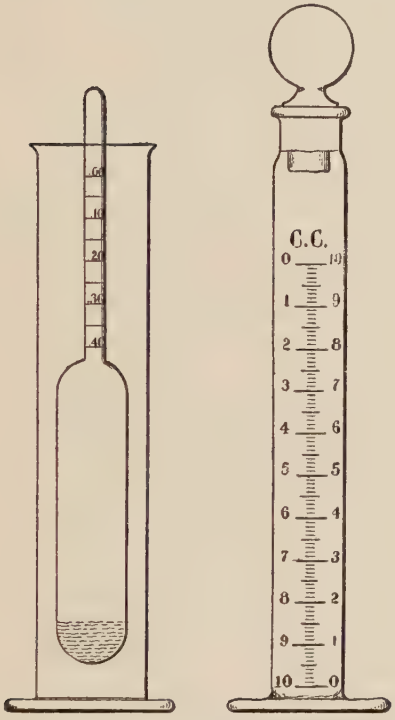


FIG. 275.—Holt's milk-testing apparatus.

FIG. 276.—Centrifuge tube for milk analysis.

Holt's apparatus, which consists of a 10-c.c. cream gage and a small hydrometer (Fig. 275). The cream gage is filled to the 0 mark with milk, allowed to stand for twenty-four hours at room temperature, and the percentage of cream then read off. The percentage of fat is three-fifths that of the cream. The protein is then approximated from a consideration of the specific gravity and the percentage of fat. The salts and sugar very seldom vary sufficiently to affect the specific gravity, hence a high specific

gravity must be due to either an increase of protein or decrease of fat, or both, and *vice versa*. With normal specific gravity the protein is high when the fat is high, and *vice versa*. The method is not accurate with cows' milk.

For more accurate work the following methods, applicable to either human or cows' milk, are simple and satisfactory:

Fat.—*Leffmann-Beam Method.*—This is essentially the widely used Babcock method, modified for the small quantities of milk obtainable from the human mammary gland. The apparatus consists of a special tube which fits the aluminum shield of the medical centrifuge (Fig. 276) and a 5-c.c. pipet. Owing to its narrow stem the tube is difficult to fill and to clean. Exactly 5 c.c. of the milk are introduced into the tube by means of the pipet, and 1 c.c. of a mixture of equal parts of concentrated hydrochloric acid and amyl-alcohol is added and well mixed. The tube is filled to the 0 mark with concentrated sulphuric acid, adding a few drops at a time and agitating constantly. This is revolved in the centrifuge at 1000 revolutions a minute for three minutes, or until the fat has separated. The percentage is then read off upon the stem, each small division representing 0.2 per cent. of fat.

Proteins.—*T. R. Boggs' Modification of the Esbach Method.*—This is applied as for urinary albumin (p. 138), substituting Boggs' reagent for Esbach's. The reagent is prepared as follows:

1.	Phosphotungstic acid	25 gm.
	Distilled water	125 c.c.
2.	Concentrated hydrochloric acid	25 "
	Distilled water	100 "

When the phosphotungstic acid is completely dissolved, mix the two solutions. This reagent is quite stable if kept in a dark glass bottle.

Before examination the milk should be diluted according to the probable amount of protein, and allowance made in the subsequent reading. For human milk the optimum dilution is 1 : 10; for cows' milk, 1 : 20. Dilution must be accurate.

Lactose.—The protein should first be removed by acidifying with acetic acid, boiling, and filtering. The copper methods may then be used as for glucose in the urine (pp. 143, 144); but it must be borne in mind that lactose reduces copper more slowly than

glucose, and longer heating is, therefore, required; and that 10 c.c. of Fehling's solution (or 25 c.c. of Benedict's) are equivalent to 0.0676 gm. lactose (as compared with 0.05 gm. glucose).

Detection of Preservatives.—Formalin is the most common preservative added to cows' milk, but boric acid is also used.

To detect formalin add a few drops of dilute ferric chlorid solution to a few cubic centimeters of the milk, and run the mixture gently upon the surface of some chemically pure sulphuric acid in a test-tube. If formaldehyd be present, a bright red ring will appear at the line of contact of the fluids. This is not a specific test for formaldehyd, but nothing else likely to be added to the milk will give it. Jorissen's test, given for formaldehyd in urine, may also be used.

To detect boric acid Goske's method, as used by the Chicago Department of Health, is simple and satisfactory: Mix 2 c.c. of concentrated hydrochloric acid with 20 c.c. of the milk and place in a 50-c.c. beaker. In this suspend a long strip of turmeric paper (2 cm. wide), so that its end reaches to the bottom of the beaker. Allow to remain about half an hour. The liquid will rise by capillarity, and if boric acid be present a reddish-brown color will appear at the junction of the moist and dry portions of the paper. When this is touched with ammonia a bluish-green slate-color develops. A rough idea of the amount of boric acid may be had by comparing the depth of color with that produced by boric acid solutions of known strength.

Bacteria.—At times, it is necessary to know the type of pathogenic organisms in a given sample of milk if it is suspected that the use of such milk has been the cause of disease. Epidemics of typhoid fever, or of a very virulent form of "streptococcic sore throat," have often been traced to a certain milk supply. Usually, however, a bacteriologic examination of milk consists of plating the milk in such a manner that an estimate can be made of the number of bacteria for each cubic centimeter. Excellent milk may have only a few hundred bacteria for each cubic centimeter. Milk with a count under 10,000 bacteria on delivery would be considered very good, and under 50,000 good. Poor milk, yet commercially usable, may have 500,000 bacteria for each cubic centimeter, while milk that should not be used may contain too many bacteria to be counted. The Standard Methods of Milk Analysis, as published by

the American Public Health Association, should be used. Briefly the technic is as follows:

Technic for Milk Counts.—1. Prepare "water blanks," sterile bottles, preferably glass stoppered. Some of these should contain exactly 99 c.c. of water, and others only 9 c.c. Sterilize in the autoclave at 15 pounds pressure for one hour.

2. Sterilize a number of 1-c.c. pipets in a hot-air oven. These are most easily handled if they are sterilized in a covered copper container in which they may be kept until ready for use.

3. Melt plain agar, slightly acid, and cool to 45° C.

4. Shake the sample of milk thoroughly, dilute as follows: (*a*) Place 1 c.c. of milk in a bottle containing 99 c.c. of sterile water; discard this pipet; (*b*) with a sterile pipet transfer 1 c.c. of the 1 : 100 dilution to a bottle containing 9 c.c. of sterile water; (*c*) with the same pipet transfer 1 c.c. of 1 : 100 dilution to a sterile Petri dish; (*d*) with a fresh sterile pipet make still another dilution by transferring 1 c.c. of 1 : 1000 dilution to a bottle containing 9 c.c. of sterile water; (*e*) with this same pipet transfer 1 c.c. of 1 : 1000 dilution to a sterile Petri dish; (*f*) finally use a fresh sterile pipet to transfer 1 c.c. of 1 : 10,000 dilution to a sterile Petri dish. All dilutions must be shaken twenty-five times before making any transfers.

5. Pour 10 c.c. of melted agar into each Petri dish. The milk dilution and the agar are thoroughly mixed by gently shaking.

6. Incubate cultures for forty-eight hours at 37.5° C.

7. Count the colonies. Only those plates are chosen in which there are not more than 300 and not less than 30 colonies in the entire Petri dish.

8. Calculate the number of organisms for each cubic centimeter from the count and the dilution; for example, there are 256 colonies in the plate; dilution 1 : 1000; number of organisms for each cubic centimeter is 256,000.

SYPHILITIC MATERIAL

In 1905 Schaudinn and Hoffmann described the occurrence of a very slender, spiral micro-organism in the lesions of syphilis. This they named *Spirochæta pallida*, because of its low refractive power and the difficulty with which it takes up staining reagents. The name was later changed to *Treponema pallidum*. Its etiologic relation to syphilis is now universally recognized. It is found in primary, secondary, and tertiary lesions, but is not present in the last stage in sufficient numbers to be of value in diagnosis.

Treponema pallidum is an extremely slender, spiral, motile thread, with pointed ends. The organism varies considerably in length, the average being about 10 to 12 μ, or somewhat greater than the diameter of a red blood-corpuscle; and it exhibits five to twelve, sometimes more, spiral curves, which are sharp and regular, and resemble the curves of a corkscrew (Figs. 277 and 278). As seen in fresh material by dark-field illumination it moves relatively slowly, forward or backward, rotating on its long axis and retaining its regular curves. It takes up stains so poorly and is so delicate that it is difficult to see even in well-stained preparations; a high magnification and careful focusing are, therefore, required. Upon ulcerated surfaces it is often mingled with other spiral micro-organisms, which adds to the difficulty of its detection. The most notable of these is *Borrelia refringens*, described on page 462.

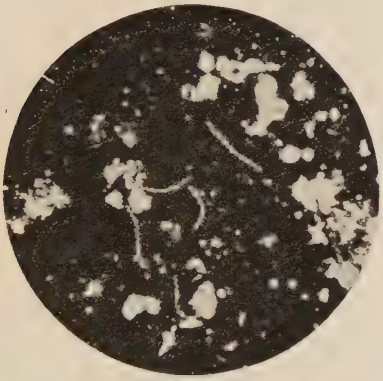


FIG. 277.—*Treponema pallidum* (× 1000) (Leitz 1/12 oil-immersion objective and Leitz dark-ground condenser). The parasite has the same appearance as in India-ink preparations.

FIG. 278.—*Treponema pallidum, Borrelia refringens*, and three red blood-corpuscles in a smear from a chancre (× 1200). From a preparation stained with Giemsa's stain, with alkali, for three hours. The treponemata were purplish red; refringens, bluish purple; red corpuscles, deep slate blue.

Treponema pallidum is most easily demonstrated in chancres and mucous patches, although the skin lesions—papules, pustules, roseolous areas—often contain large numbers. Tissue juice from the deeper portions of the lesions is the most favorable material for examination, because the organisms are commonly more abundant than upon ulcerated surfaces and are rarely accompanied by

other micro-organisms. After cleansing, the surface is gently scraped with a curet or rubbed briskly with a swab of cotton or gauze. In a few moments serum will exude and very thin smears are then made from it. Exudation of serum may be hastened by gentle squeezing or by suction with a glass tube provided with a rubber nipple. In transferring the serum from the lesion to the slide or cover-glass it is convenient to use a capillary pipet. The rubbing should not be so vigorous as to bring much blood, because the corpuscles may hide the treponema; but a few red corpuscles are an advantage as an aid in locating favorable fields and as a check upon the quality of the staining. Best fields are those with the clearest background and with a few red corpuscles, which must be well stained, well preserved, and not shrunken. If the regional lymph-nodes show enlargement the method of choice is aspiration. The skin is painted with iodin, and a stout hypodermic needle attached to a small syringe containing a few drops of sterile physiologic salt solution is inserted into the gland. Penetration of the capsule is shown by the movements of the gland when the needle is moved. The needle is rotated to break up some of the gland tissue, the salt is injected, the needle is again moved about slightly, and the fluid is withdrawn. Fluid thus obtained is often very rich in treponemata and is extremely unlikely to contain any other organism.

Methods for Treponema pallidum.—Undoubtedly the most satisfactory method for detection and microscopic study of any of the group of spirochetes is examination of fresh fluid by dark-field illumination (p. 23), but this is seldom available in the small laboratory. The practitioner will generally rely on staining methods. Of these the authors recommend Giemsa's method, and, especially, the Fontana-Tribondeau silver method.

Giemsa's stain (p. 275) is the most widely used and is one of the best (Fig. 278). It is best purchased ready prepared. Smears are fixed in absolute alcohol for fifteen minutes. Ten drops of the stain are added to 10 c.c. of faintly alkaline distilled water (1 drop of a 1 per cent. solution of potassium carbonate to 10 c.c. of the water), and the fixed smear is immersed on edge in this diluted stain for one to three hours or longer. It is then rinsed in distilled water, dried, and mounted. More intense staining may be obtained and the time shortened by conducting the process in the incubator. In well-stained specimens *Treponema pallidum*

is reddish; most other micro-organisms, bluish. If desired, Giemsa's stain may be used as described for blood (p. 275), but the organisms do not then stand out quite so clearly.

It is a waste of time to search for treponemata in films in which the leukocytes and the red corpuscles are not well stained. The nuclei of the former should be dark purple; the latter should be deep copper-red or salmon colored when the stain is used as for blood, and deep slate-blue when alkali has been added.

Wright's blood-stain, used in the manner already described (p. 272), except that the diluted stain is allowed to act upon the film for fifteen minutes, gives fair results. Medalia uses 1 per cent. sodium carbonate instead of water for diluting the Wright stain on the slide, and stains, with very gentle steaming, for twenty minutes.

Silver Method.—The silver impregnation method has long been used for tissues. It is probably best applied to smears by the *Fontana-Tribondeau method:*

1. Thoroughly dry in the air.
2. Wash several times with a solution consisting of 1 c.c. glacial acetic acid, 2 c.c. of formalin, and 100 c.c. of distilled water. Rinse gently with alcohol and flame off the excess.
3. Cover with 5 per cent. aqueous solution of tannic acid, heat until steam rises, and allow to cool for thirty seconds.
4. Rinse in water, cover with Fontana's silver solution, heat until steam rises, and allow to cool for thirty seconds.
5. Wash, dry, and mount.

Spirochetes are brown to black.

Fontana's silver solution must be freshly prepared as follows: To a 5 per cent. solution of silver nitrate in distilled water add diluted ammonium hydroxid drop by drop until a faint milky turbidity appears and remains upon shaking. Excess of ammonia must be avoided.

India-ink Method.—A small drop of India-ink of good grade (Günther and Wagner's "Chin-Chin liquid pearl" or Grübler's "nach Burri" recommended; "Bioloid Black" may also be used, and is more easily obtained) is mixed on a slide with 1 or 2 small drops of serum from the suspected lesion. The mixture is then spread over the slide and allowed to dry. After drying, it is examined with an oil-immersion lens. Microörganisms, including *Treponema pallidum*, appear clear white on a brown or black background, much as they do with the dark-ground condenser (Fig. 277). If desired, the mixture of ink and serum may be covered with a cover-glass and examined in the moist state, the living organisms being thus demonstrated. Because of its extreme simplicity this method has been favorably received. It cannot, however, be abso-

lutely relied upon, since, as has been pointed out, many India-inks contain wavy vegetable fibrils which might easily mislead a beginner, and sometimes, indeed, even an experienced worker. Instead of India-ink, collargol, diluted 1 : 20 with water and thoroughly shaken, has been recommended.

SEMEN

Absence of spermatozoa is a more common cause of sterility than is generally recognized. In some cases they are present, but lose their motility immediately after ejaculation.

Semen should be kept warm until examined. When it must be transported any considerable distance a vacuum bottle may be

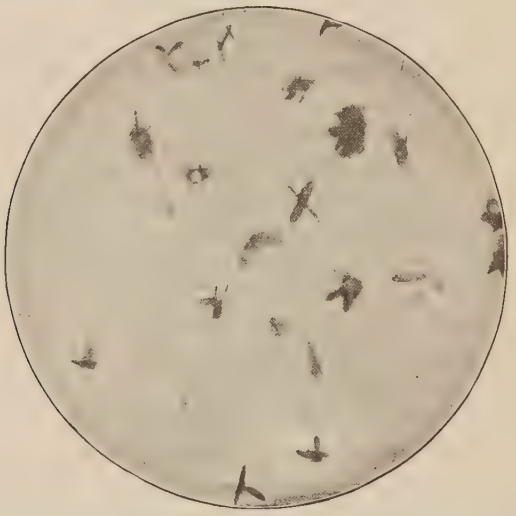


FIG. 279.—Seminal crystals (medium size) (×750) from a stain on clothing. A single thread $\frac{1}{8}$ inch long was used in the test, the stain being three years and four months old (Peterson and Haines).

used. In its absence the method suggested by Boston is convenient: The fresh semen is placed in a small bottle, to the neck of which a string is attached. This is then suspended from a button on the trousers, so that the bottle rests against the skin of the inguinal region. It may be carried in this way for hours. When ready to examine, place a small quantity upon a warmed slide and apply a cover. The spermatozoa are readily seen with a 4-mm. objective (Fig. 83). Normally, they are abundant and in active motion.

Detection of semen in stains upon clothing is often important. The finding of spermatozoa, after soaking the stain for an hour

in normal salt solution or dilute alcohol and teasing in the same fluid, is absolute proof that the stain in question is semen, although it is not possible to distinguish human semen from that of the lower animals in this way. A little eosin added to the fluid will bring the spermatozoa out more clearly, staining the heads red and the tails pink. The tails of most of the sperms will be broken off by the teasing.

Florence's Reaction.—The suspected material is softened with water, placed upon a slide with a few drops of the reagent, and examined at once with a medium power of the microscope. If the material be semen, there will be found dark-brown crystals (Fig. 279) in the form of rhombic platelets resembling hemin crystals, or of needles, often grouped in clusters. These crystals can also be obtained from crushed insects, watery extracts of various internal organs, and certain other substances, so that they are not absolute proof of the presence of semen. Negative results, on the other hand, are practically conclusive. Very rarely does the reaction fail, even when the semen is many years old.

The reagent consists of iodin, 2.54 gm.; potassium iodid, 1.65 gm.; and distilled water, 30 c.c.

Hektoen[1] has elaborated a precipitin test, which is specific not only for semen but for the particular species.

DIAGNOSIS OF RABIES

In view of the brilliant results attending prophylactic treatment by the Pasteur method, early diagnosis of rabies (hydrophobia) in animals which have bitten human beings is extremely important.

The most reliable means of diagnosis is the production of the disease in a rabbit by subdural or intracerebral injection of a little of the filtrate from an emulsion of the brain and medulla of the suspected animal. The diagnosis can, however, usually be quickly and easily made by microscopic demonstration of Negri bodies. Whether these bodies be protozoan in nature and the cause of the disease, as is held by many, or whether they be products of the disease, it is certain that their presence is pathognomonic.

Negri bodies are sharply outlined, round, oval, or somewhat irregular structures which vary in size, the extremes being 0.5 and

[1] Hektoen, L.: Specific Precipitin Test for Human Semen, Jour. Amer. Med. Assoc., vol. 78, p. 704, March, 1922.

18 μ. They consist of a hyaline-like cytoplasm, in which when properly stained one or more chromatin bodies can usually be seen. They are situated chiefly within the cytoplasm of the large cells of the central nervous system, the favorite location being the multipolar cells of the hippocampus major (Ammon's horn). In many cases they suggest red blood-corpuscles lying within nerve-cells.

Probably the best clinical method of demonstrating Negri bodies is the impression method of Langdon Frothingham, which is carried out as described below:

1. Place the dog's brain[1] upon a board about 10 inches square, and divide into two halves by cutting along the median line with scissors.

2. From one of the halves cut away the cerebellum and open the lateral ventricle, exposing Ammon's horn.

3. Dissect out Ammon's horn as cleanly as possible.

4. Cut out a small disk at right angles to the long axis of Ammon's horn, so that it represents a cross-section of the organ.

5. Place this disk upon the board near the edge, with one of the cut surfaces upward.

6. Press the surface of a thoroughly clean slide upon the disk and lift it suddenly. The disk (if its exposed surface has not been allowed to become too dry) will cling to the board, leaving only an impression upon the slide. Make several similar impressions upon different portions of the slide, using somewhat greater pressure each time. Impressions are also to be made from the cut surface of the cerebellum, since Negri bodies are sometimes present in the Purkinje cells when not found in Ammon's horn.

7. Before the impressions dry immerse in methyl-alcohol for one-half to two minutes.

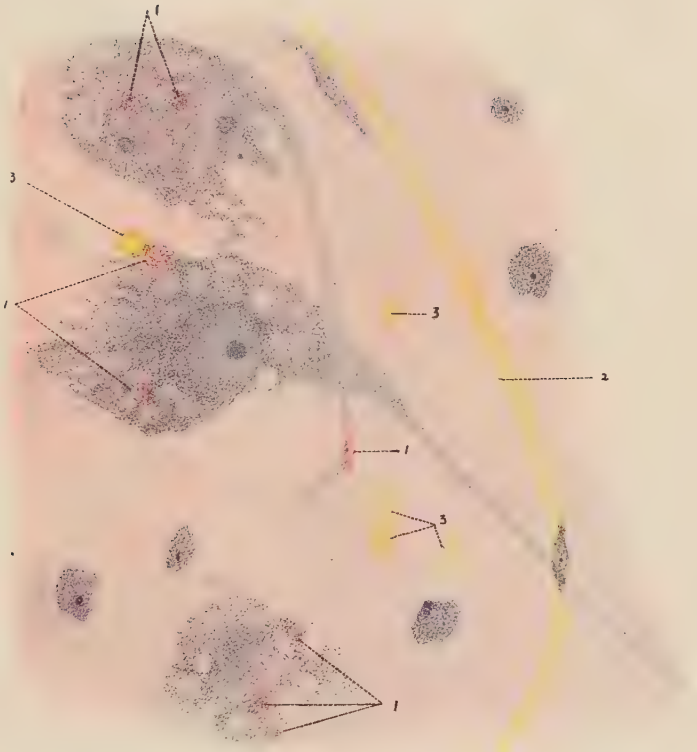
8. Cover with Van Gieson's methylene-blue-fuchsin stain, warming gently for one-half to two minutes. This stain, as modified by Frothingham, is as follows. It remains effective for three or four days:

Tap-water	20 c.c.
Saturated alcoholic solution basic fuchsin	1 drop
Saturated aqueous solution methylene-blue	1 "

9. Wash in water and dry with filter-paper. Examine with a low power to locate the large cells in which the bodies are apt to be found, and study these with an oil-immersion lens.

[1] For Dr. Frothingham's method of removing a dog's brain see American Journal of Public Hygiene for February, 1908.

PLATE XII



Nerve-cells containing Negri bodies.

Hippocampus impression preparation, dog. Van Gieson stain; × 1000. 1, Negri bodies; 2, capillary; 3, free red blood-corpuscles. (Courtesy of Langdon Frothingham.)

The Negri bodies are stained pale pink to purplish red, and frequently contain small blue dots (Plate XII). The nerve-cells are blue, and red blood-corpuscles are colorless or yellowish-copper colored.

When the work is finished the board with the dissected brain is sterilized in the steam sterilizer.

Demonstration of Negri bodies by this method is quicker and, possibly, more certain than by the study of sections. It has the decided advantage over the smear method that the histologic structure is retained. One or more of the impressions generally shows the entire cell arrangement almost as well as in sections, and it is very easy to locate favorable fields with a 16-mm. objective.

TESTS USED IN THE STUDY OF ACIDOSIS

In order to make clear the principles upon which the various tests are based it will be necessary to review some of the fundamental facts regarding the causes and nature of acidosis. Many points have only recently been cleared up, and there are still divergent views among authorities as to the precise limitations of the term "acidosis."

The never-ceasing processes of metabolism result in the continuous production within the body of a marked excess of acid radicles, chiefly from the combustion of carbon, phosphorus, and sulphur. Notwithstanding this, the hydrogen-ion concentration, or true reaction, of the blood and tissues remains slightly alkaline, close to pH 7.5.[1] This standard reaction is one of the physiologic constants of the body. Any appreciable deviation means illness, and any marked deviation is inconsistent with life.

Among the mechanisms by which the standard reaction is protected against variable amounts of acid arising in normal metabolism, and especially against the very large amounts produced in some pathologic conditions, the buffer action of the blood demands first consideration. When successive small amounts of acid are added to blood in a test-tube the reaction does not change with each

[1] The reader who is not familiar with the significance and derivation of the symbol pH, which is commonly used to indicate the hydrogen-ion concentration or true reaction of a liquid, may consult any recent work upon physical chemistry. It will suffice to state that pH 7 represents the reaction of distilled water or neutrality, and that the pH value rises with decreased hydrogen-ion concentration (alkalinity) and falls with increased hydrogen-ion concentration (acidity). Since the figure represents a logarithm, an increase or decrease of one unit represents a tenfold decrease or increase of acidity; thus, a solution whose pH is 5 is ten times as acid as one whose pH is 6.

addition of acid as would the reaction of a simple alkaline solution; the blood, in fact, seems to absorb a very considerable amount of acid before even the slightest change in reaction takes place. This "buffer action," whereby the hydrogen-ion concentration is protected against the addition of acids, is due partly to the proteins of the blood, which can act either as weak acids or weak bases, but mainly to the relatively large supply of mono- and dibasic carbonates and phosphates of the fixed bases, of which sodium is the most abundant. Each addition of acid to the blood neutralizes a corresponding amount of the base, but a compensatory readjustment of the proportions of the remaining mono- and dibasic salts takes place, whereby the original concentration of free hydrogen ions is immediately restored. Only when the reserve supply of fixed bases, sometimes called the "alkali reserve," has been largely neutralized does the buffer action fail and allow a change of hydrogen-ion concentration to take place.

It is thus the alkali reserve of the blood and tissues which most directly protects the hydrogen-ion concentration of the body fluids. This reserve is subject to marked depletion in disease, but in health is maintained at a fairly constant level, because most of the acid arising in metabolism is removed from the body by one or other of the following mechanisms:

1. A certain amount of acid is excreted by the kidneys, partly as free acid, but chiefly as acid salts, mostly acid phosphates. The power of the kidneys to secrete a frankly acid urine from a slightly alkaline blood is one of their striking and important functions.

2. A notable amount of acid is neutralized by ammonia, of which there is a constant supply derived from protein catabolism, and is excreted as ammonium salts in the urine. Such ammonia as is not thus intercepted by acids, constituting the major portion under normal conditions, is built up into urea. In certain pathologic conditions, notably in diabetes, this is an extremely important means of dealing with acids: a large part of the ammonia is diverted to this purpose and the formation of urea is correspondingly decreased.

3. Carbon dioxid, the most important acid end-product of metabolism, is eliminated chiefly in two ways: (*a*) The larger part is carried off by the lungs as follows: Carbon dioxid combines with

the normal carbonates of fixed bases which constitute the chief alkali reserve of the blood-plasma, and is carried from the tissues to the lungs in the venous blood as bicarbonate. Here the carbon dioxid passes over into the alveolar air, and the alkali again takes the form of the normal carbonate and returns to the tissues, where it again takes up carbon dioxid. (*b*) Another portion of the carbon dioxid is neutralized by ammonia and is then built up into urea and excreted by the kidneys.

The mechanisms mentioned above are adequate to meet the ordinary demands made by acids arising in normal metabolism. Only a small portion of the acid is allowed to attack the important fixed bases of the blood and tissue. This forms neutral salts which are excreted in the urine, but the lost base is soon replaced from the food, and the alkali reserve of the body thus remains undiminished. Only when the loss of base is too rapid for replacement from the food, as may occur in certain pathologic conditions, does the alkali reserve become very seriously depleted. *The clinical symptoms recognized as characteristic of acidosis then appear.* Finally, as a terminal event, the hydrogen-ion concentration of the blood may become appreciably increased.

Acidosis, then, is not the occurrence of an acid reaction in the blood or tissues; nor does it necessarily imply even the slightest reduction of the normal alkalinity as measured by hydrogen-ion concentration. It may be defined, in the words of Sellards, as "a diminution in the reserve supply of fixed bases in the blood and tissues of the body, the physicochemical reaction of the blood remaining unchanged except in very extreme conditions." For practical purposes it may be regarded as a decrease in the titratable alkalinity of the blood.

Since the fixed bases of the blood constitute the chief means of transporting carbon dioxid from the tissues to the lungs, depletion of the supply of fixed base reduces the capacity of the blood to carry carbon dioxid. This leads to accumulation of carbon dioxid in the tissues and consequent blocking of the processes of oxidation, so that the individual suffers from asphyxia exactly as if he were deprived of air. The respiratory center is stimulated, leading to increased pulmonary ventilation, which, when marked, becomes hyperpnea or air hunger, a most characteristic clinical sign of acidosis.

Conditions in which acidosis might occur may, following Macleod, be outlined as follows:

I. Increase of acids in the body.
   1. Excessive formation of acids.
      (*a*) Excessive formation of beta-oxybutyric and diacetic acids from defective oxidation of fats in disturbances of carbohydrate metabolism or in carbohydrate starvation. This is a common and important form of acidosis, sometimes called "ketosis," and is best seen in diabetes mellitus.
      (*b*) Excessive decomposition of proteins, as in fevers.
      (*c*) Formation of acid in excessive intestinal fermentation.
   2. Accumulation of acids because of defective elimination.
      (*a*) Accumulation due to diminished ability of the kidneys to excrete acid, as in chronic interstitial nephritis.
      (*b*) Accumulation of carbon dioxid in asphyxial conditions.
   3. Administration of acids. Large therapeutic doses of hydrochloric acid may cause acidosis of sufficient degree to be detected by certain of the laboratory tests.

II. Decrease of base. There may be a primary loss of alkali reserve by abstraction of fixed base, as in very severe diarrheal conditions, pancreatic and biliary fistula, and so forth. Lack of sufficient base in the food to restore the normal loss might conceivably be a factor in some cases.

Clinically, acidosis of sufficient degree to have any serious significance occurs in only a few conditions: diabetes mellitus, in which the acidosis is due to excessive production of beta-oxybutyric and diacetic acids; acute nephritis and advanced chronic interstitial nephritis, in which there appears to be accumulation of acid from failure of the kidneys to excrete acids normally; Asiatic cholera, in which the acidosis is due partly to abstraction of base, partly to coexistent nephritis; and certain diarrheal conditions of childhood, in which the cause of the acidosis is uncertain.

Milder grades of acidosis without serious clinical significance may occur in a variety of conditions, notably acute rheumatic fever, advanced cachexias, and severe anemias.

It must also be borne in mind that there may occur a condition that is the opposite of acidosis, namely, "alkalosis." In these patients the CO_2-combining power of the blood is high. The condition is particularly important when renal function is impaired, or in pyloric obstruction when tetany may occur. The administration of sodium bicarbonate is, of course, decidedly contraindicated in such a condition.

Tests for Acidosis.—The characteristic clinical sign of acidosis is hyperpnea or air hunger, but this is a comparatively late symptom. The state of acidosis can be diagnosed by laboratory means long before definite clinical symptoms develop. The various laboratory tests are based upon one or another of the facts regarding the cause and nature of acidosis which have been mentioned in the previous pages and may be classified as follows:

I. *Tests which measure the hydrogen-ion concentration of the blood.* None of these need be given here. While an increase of hydrogen-ion concentration is indeed very definite evidence of acidosis, yet, as has been shown in the previous pages, the buffer action of the carbonates, phosphates, and proteins of the blood is so effective that hydrogen-ion concentration changes only when the acidosis is very severe.

II. *Tests which measure the alkali reserve*—practically the buffer power of the blood and tissues—either directly or indirectly. Since acidosis is essentially a depletion of this reserve, these tests are theoretically the best and most generally applicable, provided the technical methods are satisfactory.

1. Titratable alkalinity of the blood. No simple and accurate method is available.

2. The bicarbonate tolerance test of Sellards, given in detail on page 91. This consists in finding the amount of sodium carbonate which can be given by mouth or intravenously without causing the urine to become alkaline to litmus. The tolerance of a normal individual is about 3 to 5 gm.; in acidosis it may reach 100 to 150 gm. It is assumed that this amount is retained in the body in order to restore the depleted reserve. This is one of the most satisfactory clinical tests for acidosis, for it is applicable to all forms, is sufficiently sensitive to detect the slight grades which produce no clinical symptoms, furnishes a very definite measure of the degree of acidosis, and at the same time supplies the approved treatment.

3. The carbon dioxid carrying power of the blood. Carbon dioxid is carried to the lungs chiefly in combination with the fixed bases which are the principal buffer substances of the blood. When these are diminished the capacity of the blood to carry carbon dioxid is correspondingly diminished. The carbon dioxid combining power of the blood-plasma is then a useful measure of the reserve of fixed bases in the blood, and may be determined by the method of Van Slyke and Cullen, described on page 351. This is probably the most reliable test for acidosis and is applicable to all forms.

4. Carbon dioxid tension of alveolar air. The percentage of carbon dioxid in the expired air depends upon the power of the

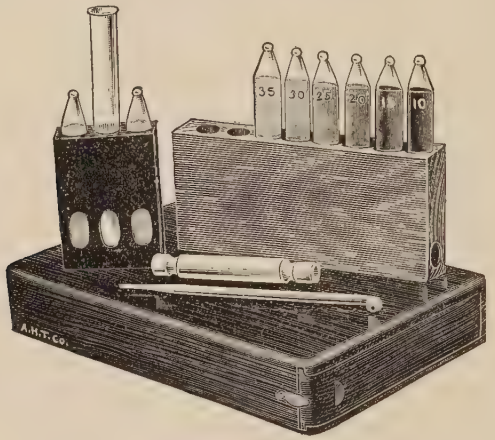


Fig. 280.—Marriott's alveolar air-testing outfit.

blood to transport carbon dioxid, that is, upon the supply of fixed bases, and, other things being equal, varies directly with it. Other factors which influence the carbon dioxid tension of expired air are increased pulmonary ventilation and changes in the lungs which interfere with the exchange of gases. The former is best illustrated by the effect of high altitudes, where, as Yandell Henderson has remarked, all inhabitants suffer from acidosis if this test be taken as the sole criterion. The simplest method for determining the percentage of carbon dioxid in alveolar air is that of Marriott.

Marriott's Method for Carbon-dioxid Tension of Alveolar Air.—This is based upon the change in reaction and corresponding change in color of a standard carbonate solution containing the indicator phenol-

sulphonephthalein when the alveolar air is passed through it, the degree of change depending upon the tension of carbon dioxid in the air regardless of the amount of air which passes through the solution. The color change is measured by comparison with a series of color standards of known hydrogen-ion concentration.

The complete outfit (Fig. 280) for this method may be purchased ready for use.[1] It consists of a set of 8 neutral-glass tubes filled with standardized color solutions and numbered 10, 15, 20, 25, 30, 35, 40, 45, respectively; a long test-tube; a capillary tube, and a comparison box. These are contained in a small wooden box. The outfit also includes a flask of indicator solution, a rubber bag of about 1000 c.c. capacity, with mouthpiece and pinch-cock, and a rubber inflation bulb. It is accompanied by full directions for use.

The reading is in terms of millimeters of mercury. In normal adults this generally falls between 40 and 45 mm.; in mild acidosis, 30 to 35 mm.; in severe acidosis, below 20 mm.

III. *Tests which show excessive or abnormal formation of acids within the body.* When such is demonstrated acidosis or, at least, the tendency to acidosis is assumed.

1. Detection of abnormal acids. This is practicable only in the case of beta-oxybutyric and diacetic acids, which, together with the derivative acetone, can be detected in the urine (p. 153).

2. Detection of excessive elimination of acids, which presupposes excessive production.

(*a*) By quantitative estimation of ammonium salts in urine (p. 127). This is of much value in diabetes where a large proportion of the abnormal acid is neutralized by ammonia and excreted as ammonium salts in the urine. For some reason, not yet clear, ammonium salts of the urine are not increased in the acidosis of nephritis. In this connection the recent work of Nash and Benedict, indicating that formation of ammonia is a function of the normal kidney, is interesting. It must not be forgotten that other conditions, such as organic disease of the liver, which decrease the formation of urea, may increase ammonium salts in the urine.

(*b*) By titrating the total acidity of the urine (p. 90). This gives a rough index of acidosis, but has little practical value because the ability of the kidney to secrete an excess of acid is limited, especially in nephritis.

[1] This is manufactured by Hynson, Westcott & Dunning, Baltimore.

CAPILLARY MICROSCOPY

The principles of microscopic study of the capillaries were first laid down by Lombard in a study of the blood-pressure in arterioles. If a drop of transparent oil, such as cedarwood oil, is placed on the skin and examination made under a low-power microscope by a strong light, a remarkable view of the superficial terminal vessels may be obtained. Recently considerable attention was paid to this subject, which started as a physiologic study, as it has some clinical application in such conditions as polycythemia and Raynaud's disease. The studies are directed toward form, size, tonus, flow, and reaction to stimuli.

Several attempts have been made to utilize photography. The problems of sufficient light for quick exposures, prevention of movement, elimination of heat and actinic rays, and accurate focusing

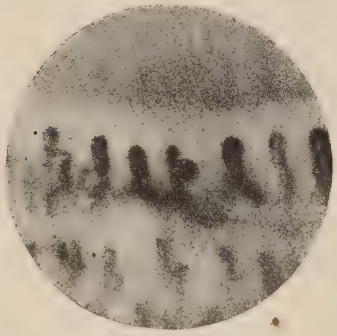


FIG. 281.—Normal capillaries (× 50) (Sheard).

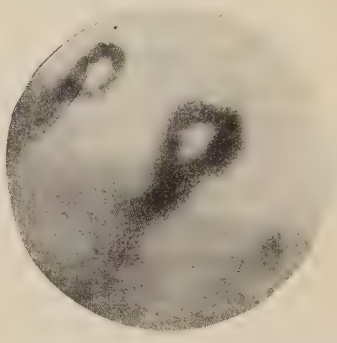


FIG. 282.—Capillaries in Raynaud's disease (× 50) (Sheard).

have all had to be met. Some of the best photographs have been obtained by a method described by Sheard (Figs. 281 and 282). The student is referred to the literature[1] for further details on this interesting subject which may prove to be of considerable value in studying certain rather rare diseases.

[1] Sheard, Charles: Instantaneous Photomicrography of the Skin Capillaries in the Living Human Body, Science, vol. 60, p. 409, October, 1924. Sheard, Charles, and Brown G. E.: A Method for Instantaneous Photomicrography of the Skin Capillaries, Jour. Lab. and Clin. Med., vol. 10, p. 925, August, 1925. Callander, C. L.: Photomicrographic Studies of Morphology of Surface Capillaries in Health and Disease, Jour. Amer. Med. Assoc., vol. 84, p. 352, January, 1925. Giffin, H. Z., and Brown, G. E.: Studies of the Vascular Changes in Cases of Polycythemia Vera, Amer. Jour. Med. Sci., vol. 171, p. 157, February, 1926.

CHAPTER IX

SERODIAGNOSTIC METHODS

PRELIMINARY CONSIDERATIONS

When an individual suffers from an infectious disease there are formed by his tissues certain substances which possess the power to destroy or otherwise injuriously affect the disease-producing agent, or to neutralize its poisons. They reside in the blood-plasma and lymph, and appear in the serum when the blood coagulates. They may appear in the blood very soon after the individual is taken sick, or they may be delayed until the disease is well advanced; and they may remain for a variable time after his recovery.

These substances, called "antibodies" or "immune bodies," are *specific*, that is, each is produced only as a result of the activity within the body of a particular disease-producing agent, or "antigen," and it acts antagonistically against this particular antigen alone. Therefore the presence of any of these immune bodies in an individual's blood may, with certain exceptions to be noted later, be regarded as pathognomonic of the corresponding disease, and the search for them may be resorted to for diagnostic purposes whenever they can be found more easily than can the disease-producing agent. Upon this foundation has grown up a long series of diagnostic procedures, some simple, some very complicated, which play an important and growing part in medicine under the name "serodiagnosis."

With one exception the tests described in this chapter belong in this catagory, and it is therefore necessary to give such definitions of the immune bodies concerned as may enable the reader to undertake the tests with a reasonably intelligent conception of their mechanism. The mode of formation, structure, and action of the antibodies are customarily described in terms of Ehrlich's "side-chain theory of immunity" which divides them into three "orders."

Immune Bodies of the First Order.—These are antibodies with one combining portion only. They seize upon the antigen, which in this case is a toxin, while it is still free in the blood and lymph, in such a manner as to leave it no unsaturated affinities by which it may combine with the tissues. The toxin is thus rendered harmless. This order of immune bodies includes only the antitoxins, for example, those of diphtheria and tetanus. They are extremely important in medicine, but are not utilized for diagnosis.

Immune Bodies of the Second Order.—These have a combining portion similar to that of the first order, and, in addition, a portion possessing a ferment-like action, by means of which the characteristic action of the body is effected. The ferment, or zymophore, portion is readily destroyed by heat, so that serum to be used for any of the purposes included in the group must not be heated. The group includes the agglutinins, responsible for the several applications of the Widal reaction; the precipitins, responsible for one of the biologic methods to be described later for the identification of blood-stains, and the opsonins.

Immune Bodies of the Third Order.—These antibodies consist of two combining affinities only. One of these combines with the antigen, the other combines with a substance which is called complement because it "complements" or supplements or completes the specific action of the immune substance. Complement is normally present in the blood, but is unable to act upon the antigen without the mediation and aid of the immune body. The latter is, therefore, called the amboceptor. It is relatively thermostabile and keeps for a long time under suitable conditions. It is to be remembered that this is the specific immune substance whose presence or absence is indicative of the presence or absence of the corresponding disease. The native, normally present complement is relatively thermolabile, being destroyed in a few minutes by a temperature of 54° to 56° C., and keeps only a few days under the best conditions. It is non-specific, and within certain limits the complement of one species may be substituted for that of another.

This group includes the bacteriolysins and hemolysins which are utilized in the various applications of the complement-fixation method to the diagnosis of syphilis (Wassermann reaction), gonorrhea, tuberculosis, and echinococcus disease.

I. REACTIONS BASED UPON IMMUNE BODIES OF THE SECOND ORDER

A. The Agglutinins

Agglutination tests may be employed for the diagnosis of a variety of infections—typhoid, paratyphoid, bacillary dysentery, the plague, Asiatic cholera, epidemic meningitis, and others. In clinical work it is used chiefly for the diagnosis of typhoid and paratyphoid infections, and is then known as the Widal reaction. In most of the other diseases mentioned the test reacts positively only late in the disease, sometimes only after recovery.

1. The Widal Test.—(1) **Materials Required.**—1. A homogeneous suspension of bacteria of the species suspected of causing the disease, in this case typhoid or paratyphoid bacilli.

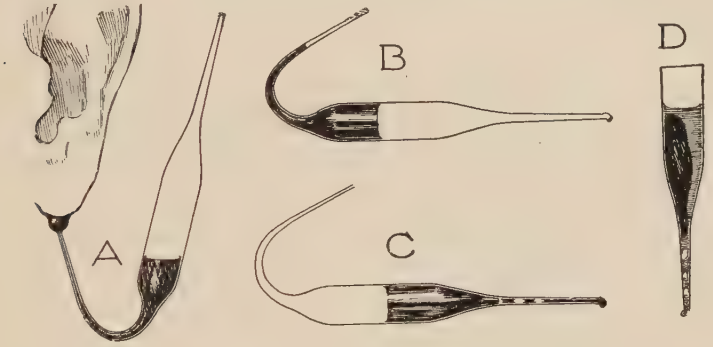


Fig. 283.—Method of obtaining blood in a Wright capsule: A, Filling the capsule; the long arm should be held more nearly horizontal than is here represented; B, the bulb has been warmed and the capillary end sealed in the flame; C, cooling of the capsule has drawn the blood to the sealed end; D, the serum has separated, and the top of the capsule has been broken off.

For the *macroscopic method* suspensions of killed organisms are used, and these may be purchased from the manufacturers of biologic preparations or may be prepared by the worker himself. In the latter case one uses a twenty-four-hour bouillon culture of a strain of the micro-organism which has been attenuated by at least several weeks' growth on culture-media, with frequent transplantation. To each 10 c.c. of the bouillon culture is added 0.1 c.c. of a 10 per cent. solution of the standard 40 per cent. formaldehyd. The culture is then placed in the ice-box, and will be sterile in three or four days. It should be shaken vigorously several times a day. If any clumps remain, the suspension should be filtered through a layer of sterile absorbent cotton. Such a suspension will keep for

many months. Whenever any tendency to spontaneous agglutination becomes evident it must be discarded.

For the *microscopic method* living bouillon cultures of eighteen to twenty-four hours' growth are employed.

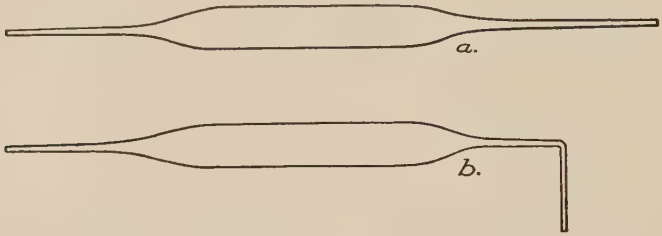


FIG. 284.—A satisfactory glass capsule for obtaining small quantities of blood, as for the Widal test. The straight tube (*a*) is more convenient to carry in the hand-bag than is Wright's capsule. It may be bent as shown in the lower figure by brief application of a match flame at the bedside. After the tube is filled the ends may be sealed with the match flame.

2. About 0.1 c.c. of the *patient's serum*. This may be obtained by pricking the cleansed finger or ear rather deeply, and collecting 10 or 15 drops of blood in one of the capsules shown in Figures

FIG. 285.—A very satisfactory blood-capsule described by Lyon. A short section of glass tubing is heated and drawn out as in making a capillary pipet; the large end is sealed in the flame; and after it has cooled, the capillary tip is sealed. The side of the tube near the capillary end is then held in the edge of a flame until it softens and a "blow-hole" is formed through expansion of the contained air. The tip of the capillary portion is then broken off, and the capsule is filled as shown in the figure, the "blow-hole" allowing the blood to enter freely.

283–285. More than one capsule should be at hand, so that a fresh one may be substituted should the first be plugged by fibrin before enough blood is obtained.

Sufficient blood may also be collected by allowing drops to dry

on glass or unglazed paper (without heating), to be afterward macerated in water. In this case, however, dilutions can only be made approximately.

3. *Physiologic saline.*

4. *Small test-tubes*, about 8 mm. in diameter, are required for the macroscopic test. These may be made from short sections of glass tubing by sealing one end in a flame.

5. *Slides*, preferably hollow ground, *cover-glasses*, and *vaselin*, are required for the microscopic test.

(2) **Methods.**—Two methods of performing the test, the macroscopic and the microscopic, will be described. The former is probably preferable:

(1) **Macroscopic Method.**—Separate the clot and serum by centrifugation, nick the wall of the capsule a short distance above the serum with a file, and break the capsule at this point. Pipet off the serum, place it in a clean test-tube, and add 9 volumes of salt solution. Counting the drops of serum as they fall from the capillary pipet, and adding nine times the number of drops of salt solution will give sufficiently accurate dilution. Now place seven small test-tubes in a rack, and add to each one, *except the first*, 0.5 c.c. of physiologic saline by means of a serologic pipet. Then place in the first and second tubes *only* 0.5 c.c. of the diluted blood-serum. Shake the second tube, and with the pipet transfer 0.5 c.c. to the third tube. Shake this tube, and transfer 0.5 c.c. to the fourth tube, and so on, to the next to the last tube, from which 0.5 c.c. should be discarded. The last tube serves as a control and should contain only 0.5 c.c. of salt solution, without any serum. In the absence of a graduated pipet the quantities may be measured by counting drops from a medicine-dropper. One thus arrives at a series of dilutions of the serum, as follows: 1 : 10, 1 : 20, 1 : 40, 1 : 80, 1 : 160, 1 : 320. Now add to each tube 0.5 c.c. of the suspension of killed bacteria. This doubles the dilution of the serum in each of the tubes. Mix all the tubes thoroughly by shaking, and place the rack in a moderately warm place or in the incubator for eight to twelve hours. In those tubes in which the reaction is positive there will be found a sediment consisting of agglutinated bacteria at the bottom of the test-tube, with a clear supernatant fluid. The control tube and the negative tubes will be cloudy and without sediment.

Dead cultures of typhoid and paratyphoid bacilli, together with all apparatus necessary for performing the macroscopic test, are put up at moderate cost by various firms under such names as typhoid diagnosticum and typhoid agglutometer. Full directions accompany these outfits.

Bass and Watkins have described a modification of the macroscopic method (using very concentrated suspensions of the bacilli) by which the test can be applied at the bedside. Agglutination occurs within a few minutes. The apparatus has been put upon the market by Parke, Davis & Co.

(2) **Microscopic Method.**—Arrange a series of dilutions of the blood-serum as above, or, if dried blood be used, macerate the dried clot with salt solution or tap-water. In the latter case, unless the size of the original drop of blood be known, the color is the only guide as to the degree of dilution. A light amber color will roughly correspond to a dilution of 1 : 50. From such a dilution others can be prepared. On the center of each of several clean cover-glasses place a loopful of each of the several dilutions, employing a platinum loop of about 2 mm. diameter. With the same loop add to each droplet of diluted serum a loop from a twelve- to twenty-four-hour-old bouillon culture of the organism in question, or of a suspension in salt solution prepared from a young agar-slant culture. This doubles the dilution of serum in each case. One cover-glass containing no serum should be prepared to serve as a control. Press over each cover-glass a hollow-ground slide previously ringed with vaselin. Turn the slide over so as to bring the cover-glass on top. Drying is prevented and the cover-glass held in place by the vaselin.

When hollow-ground slides are not at hand, a drop each of the diluted serum and the bacterial suspension may be placed in the center of a ring of vaselin on an ordinary slide and a cover-glass applied to this. Vaselin containing an antiseptic must not, of course, be used for this purpose.

Place the slides in a moderately warm place or in the incubator at 37° C. for two hours. Examine under the oil-immersion lens or, better, the high-power dry lens of the microscope, *using very subdued light.* In the negative slides and in the control the organisms

will be found moving freely and not clumped, while in the positive slides the organisms are found motionless and gathered in tangled masses and balls, that is, they are agglutinated (Fig. 286). Pseudo-reactions, in which there are a few small clumps of organisms whose motion is not entirely lost, together with many freely moving organisms scattered throughout the field, should not mislead.

Jagic suggests that, after agglutination has taken place, a drop of the suspension be mixed with a drop of India-ink and spread upon a slide. In this way a permanent record may be kept. To insure sterility the preparation may be fixed by heat.

(3) **Interpretation of Results.**—In recording the result of a Widal test it is necessary to state the highest dilution at which definite agglutination occurred, and also the time required, count-

FIG. 286.—Showing clumping of typhoid bacilli in the Widal reaction. At one point a crenated red blood-corpuscle is seen (Wright and Brown).

ing from the first mixture of bacilli and serum. Experience has shown that not much significance attaches to reactions occurring in two hours with dilutions of serum less than 1 : 40 or 1 : 80. A number of other disease conditions may occasionally give rise to a positive reaction with the typhoid bacillus, notably infections with closely related organisms, such as the paratyphoid and colon bacilli. In such cases, if tests are made with several species, the species agglutinated in the highest serum dilution may be regarded as the cause of the infection.

Following antityphoid vaccination the Widal reaction becomes positive and remains so for at least a year, and in about 25 per cent. of the cases for four or five years, or longer. A single Widal test is, therefore, of no value for diagnosis if the patient has received

antityphoid vaccination within several years previously. However, a series of positive reactions of increasing strength from day to day may be accepted with confidence.

In typhoid fever the average time of first appearance of the reaction is the eighth or tenth day of recognizable illness, although, exceptionally, it may appear as early as the third or fourth day. In doubtful cases the test should be repeated at frequent intervals, and no disappointment should be felt if, as sometimes, though rarely, happens, the reaction does not appear until the third or fourth week. It is evident, therefore, that its value for early diagnosis is much less than that of the blood-culture (p. 306). After the Widal reaction first appears it remains throughout the whole course of the disease and often persists for many years.

2. Agglutination Method in Identification of Bacteria.—The agglutination test may also be used as a means of differentiating certain bacterial species, a suspension of the organisms being mixed with ascending dilutions of blood-serum of an animal which has been immunized against the species in question. If agglutination occurs in high dilution, the organism is of the same species as that which was used for the immunization. Recently isolated cultures are, however, more resistant to agglutination than old attenuated ones. The technic is that given for the Widal test, the macroscopic method being generally the more satisfactory. The agglutination method is most frequently used to establish the identity of suspected typhoid and paratyphoid bacilli recovered from blood, urine, or feces, for the meningococcus when found in throat cultures, and for determining the types of the pneumococcus. Immune serum for these purposes can be secured from the biologic supply houses.

B. The Precipitins

These may be utilized for many purposes, notably for the biologic identification of unknown proteins, for diagnosis of echinococcus disease, and for determining the types of the pneumococcus.

1. Biologic Identification of Unknown Proteins.—This is applied chiefly to the differentiation of human and animal blood for medicolegal purposes.

(1) **Materials Required.**—The following equipment is needed:

1. *Blood-serum* of an animal (rabbit) highly immunized against

the protein to be determined. Immunize several rabbits by intravenous or intraperitoneal injections of a sterile solution of the desired protein, for example, human blood, or, better, blood-serum. The blood may be obtained from a vein. One injection of 30 c.c. of serum followed by bleeding in twelve days has given good results in our laboratory, but a considerable percentage of the rabbits die. For a slower method, which is preferable, the doses may be given at five-day intervals, two doses of 8 c.c. each, two of 5 c.c., and two of 3 c.c. To prevent anaphylactic shock the rabbit may be desensitized by injecting 0.2 c.c. of the serum one-half hour before injection of each of the later doses. After the third or fourth dose draw 2 or 3 c.c. of blood from an ear vein, separate the serum, and determine its strength as follows:

Prepare dilutions of (in this case) human blood-serum in the proportions 1 : 1000, 1 : 5000, 1 : 10,000, 1 : 20,000, and so forth, using physiologic salt solution as a diluent. Place 0.5 c.c. of the several dilutions in each of a series of very small test-tubes. By means of a capillary pipet, place 0.1 c.c. of the rabbit's serum at the bottom of each of the tubes so as to form a sharp line of contact between the two fluids. A distinct white cloud should appear at the line of contact in the lowest dilution (1 : 1000) within a minute or two, rapidly deepening to a flocculent precipitate; the reaction develops more slowly in the higher dilutions, but no reaction is significant which is not definite within twenty minutes.

If the titration results as above described, anesthetize the rabbit *while it is in a fasting condition*, as otherwise the serum is likely to be opalescent; remove the anterior breast wall under aseptic conditions; take out the left lung, and open the heart, so as to allow the animal to bleed to death into its pleural cavity. Cover the body with sterile towels wet with antiseptic solution. After clotting has occurred, pipet the serum into sterile bottles and add one-tenth its volume of 5 per cent. phenol as a preservative. If the serum is opalescent it cannot be used; if cloudy it must be filtered clear through a sterile Berkefeld filter. Sometimes the cloudiness can be removed by simple centrifugation. The titration above described should be repeated and verified before the serum is used for making the test proper.

Precipitating sera for the proteins of horse, dog, sheep, beef, fowl, etc., may, of course, be prepared in the same way.

2. *A solution of the unknown substance in physiologic salt solution.* The stock dilution should be about 1 : 1000. If made from a dried clot this can only be approximate. The solution must be made perfectly clear—by filtration if necessary—and should meet the following conditions:

(*a*) It should be almost completely colorless by transmitted light.

(*b*) It should give only a slight cloudiness when heated with a little nitric acid.

(*c*) It should, nevertheless, foam freely on shaking.

(*d*) It should be neither strongly acid nor strongly alkaline to litmus. Very weak sodium hydroxid or hydrochloric acid may be used for neutralization.

(2) **Method.**—Arrange a series of 7 small test-tubes and charge them as follows, placing the second fluid beneath the first by means of a capillary pipet as was described for the titration (p. 579).

Tube No. 1.—0.5 c.c. of the solution of the unknown protein, diluted 1 : 1000, plus 0.1 c.c. of the immune serum.

Tube No. 2.—0.5 c.c. of the unknown solution, diluted 1 : 1000, plus 0.1 c.c. of normal rabbit serum.

Tube No. 3.—0.5 c.c. of physiologic salt solution plus 0.1 c.c. of the immune serum.

Tube No. 4.—0.5 c.c. of a 1 : 1000 dilution of known serum of the species suspected to be present in the unknown material plus 0.1 c.c. of the immune serum.

Tube No. 5.—0.5 c.c. of a 1 : 1000 dilution of serum of a species different from that suspected to be present in the unknown material plus 0.1 c.c. of the immune serum.

Tube No. 6.—0.5 of the unknown solution alone.

Tube No. 7.—If the solution of the unknown protein was made from a stain upon cloth, leather, or other material, this tube should be set up. It contains 0.5 c.c. of a salt solution extract of the material plus 0.1 c.c. of the immune serum.

When the first and the fourth tubes give a definitely positive reaction, as indicated by a distinct whitish ring at the zone of contact of the two fluids, and all the other tubes give a negative reaction, the presence of protein of the species tested for is established. When only a limited amount of material is available the test can be made in capillary tubes.

(3) **Interpretation of Results.**—The precipitin reaction is closely specific, and is fully established for medicolegal purposes. Here it is generally a question of the identity of blood-stains on

clothing or implements, and it must be remembered that the test does not prove the presence of *blood*, but only of a protein of the species indicated. Doubt can arise only between the proteins of very closely related species, as, for example, man and monkey, sheep and goat, etc., and this can practically always be removed by the use of adequate controls. The power of proteins to react with precipitin may be reduced or destroyed by alcohol, formaldehyd, strong acids and alkalies, great heat, and decomposition. Dried blood is much more resistant to these influences than is blood in the fluid state.

2. Other Precipitin Tests.—The precipitins are very useful in diagnosis of echinococcus disease. The test may be applied in the manner described above. The two fluids required are fluid from a hydatid cyst, which contains the precipitable substance, and blood-serum of the patient, which contains the specific precipitin if he be suffering from echinococcus disease. Both fluids are used undiluted. The test may also be applied by mixing equal parts of the fluids in a test-tube. A positive reaction is indicated by the appearance of a flocculent precipitate within one-half hour. Control tests should be carried out with normal serum, and, when possible, with serum from a known case of hydatid disease.

For determining pneumococcus types in sputum, when the organism cannot readily be obtained in sufficiently pure culture to allow of agglutination tests, the precipitin test may be applied as described in the section upon Pneumococcus Typing (p. 666). Similar methods have lately been used for identifying diphtheria bacilli in swabs and cultures and typhoid bacilli in feces.[1]

C. The Opsonins

That phagocytosis plays an important part in the body's resistance to bacterial invasion has long been recognized. According to Metchnikoff, this property of leukocytes resides entirely within themselves, depending upon their own vital activity. The studies of Wright and Douglas, upon the contrary, indicate that the leukocytes are impotent in themselves, and can ingest bacteria only in the presence of certain substances which exist in the blood-plasma. These substances have been named *opsonins*. They prob-

[1] For diphtheria see Smith, G. H., and Kaufman, C. E.: Jour. Lab. and Clin. Med., vol. 7, p. 619, July, 1922; for typhoid see Laird, J. L., Conover, J. R., and Butts, D. C. A.: Amer. Jour. Med. Sci., vol. 165, p. 241, February, 1923.

ably act by uniting with the bacteria, thus preparing them for ingestion by the leukocytes; but they do not cause death of the bacteria, nor produce any appreciable morphologic change. They appear to be more or less specific, a separate opsonin being necessary to phagocytosis of each species of bacteria. There are, moreover, opsonins for other formed elements—red blood-corpuscles, for example. It has been shown that the quantity of opsonins in the blood can be greatly increased by injection of dead bacteria.

To measure the amount of any particular opsonin in the blood Wright has devised a method which involves many ingenious and delicate technical procedures. Much skill, such as is attained only after considerable training in laboratory technic, is requisite, and there are many sources of error. It is, therefore, beyond the province of this work to recount the method in detail. In a general way it consists in: (*a*) Preparing a mixture of equal parts of the patient's blood-serum, a suspension of the specific micro-organism, and a suspension of washed leukocytes; (*b*) preparing a similar mixture, using serum of a normal person; (*c*) incubating both mixtures for a definite length of time; and (*d*) making smears from each, staining, and examining with an oil-immersion objective. The number of bacteria which have been taken up by a definite number of leukocytes is counted, and the average number of bacteria per leukocyte is calculated; this gives the "phagocytic index." The phagocytic index of the blood under investigation, divided by that of the normal blood, gives the *opsonic index* of the former, the opsonic index of the normal blood being taken as 1. Simon regards the percentage of leukocytes which have ingested bacteria as a more accurate measurement of the amount of opsonins than the number of bacteria ingested, because the bacteria are apt to adhere and be taken in in clumps.

II. REACTIONS BASED UPON IMMUNE BODIES OF THE THIRD ORDER

The reactions of this group comprise the various applications of the Bordet-Gengou phenomenon of complement fixation. The principle may be applied to the diagnosis of any disease the antigen of which is known and obtainable in suitable form. The list includes syphilis, tuberculosis, gonorrhea, echinococcus and cysticercus disease, trichiniasis, typhoid fever, and pneumococcus, streptococcus, meningococcus, and staphylococcus infections. In several of these other and simpler methods of diagnosis are, however,

available; and in some others the complement-fixation method is not sufficiently reliable to be of value in clinical work. The method as applied to the first three of the diseases above mentioned is given in the following pages. It is most useful in syphilis.

The mechanism of the complement-fixation reactions will be made clear by a review of the processes of bacteriolysis and hemolysis, upon which they are based.

Bacteriolysis.—In 1894 Pfeiffer, by his classical experiments with cholera spirilla placed in the peritoneal cavities of guinea-pigs which had recovered from cholera, definitely established the general principle that when an animal becomes actively immune to a disease, either experimentally or by contracting the disease and recovering in the natural way, its blood-serum and tissue juices thereby acquire strong powers of killing and sometimes dissolving bacteria of the kind causing that particular disease, while they are relatively harmless to other kinds of bacteria. Such destruction of bacteria is known as bacteriolysis. It was later shown that this power is not due to a single substance in the immune animal's serum, but to two substances which act in combination, neither one being capable of causing bacteriolysis by itself. One of these exists only in the blood and tissue juices of the immune animal, and is relatively resistant to heat (thermostabile) and to drying. The other is present in the blood-serum of nearly all warm-blooded animals, whether immunized or not, and is very unstable and easily destroyed outside the body, especially by heating (thermolabile). Ehrlich explained the origin and mode of action of these two substances by his well-known side-chain theory, and named them "amboceptor" and "complement" respectively. To the bacterium, whose presence has induced the immunity and against which the newly acquired bacteriolytic activity is directed, the name "antigen" is given. According to Ehrlich the bacteriolytic power really resides in the complement, the specific amboceptor merely serving as an intermediate body or connecting link which binds the complement to a particular kind of bacterium, and thus enables the complement to act. Whenever this union of the three bodies takes place—whether within the body of an animal or in a test-tube—bacteriolysis results. Should the appropriate amboceptor or bacteriolysin be absent, complement, even if abundantly present, cannot be bound to the bacteria, and hence does not attack them.

If, upon the other hand, the complement is absent, union of the amboceptor and bacterin does, indeed, take place, but bacteriolysis does not occur. In such cases the bacteria are said to be sensitized, and subsequent addition of complement will quickly bring about bacteriolysis.

The process of bacteriolysis can be followed by careful microscopic study of the disintegrating bacteria, but is not visible to the unaided eye.

Hemolysis.—Many structures other than bacteria can act as antigens. Among these are various body cells, notably red blood-corpuscles, whose destruction is known as *hemolysis*. The mechanism is entirely analogous to that just described for bacteriolysis: injection of washed red blood-corpuscles from another species of animal induces the formation of hemolytic amboceptor, or hemolysin, which is capable of binding complement to red corpuscles of that species and of thus bringing about their destruction.

The process of hemolysis when carried out in a test-tube is easily followed with the unaided eye because of the liberation of hemoglobin from the damaged corpuscles. When the reagents are first mixed the corpuscles form an opaque reddish suspension. As hemolysis proceeds, their hemoglobin diffuses out through the fluid, which finally assumes a clear, transparent red color with no visible sediment. Should hemolysis fail to occur the intact corpuscles slowly settle to the bottom, forming a red sediment with a clear, colorless fluid above it.

In order to avoid confusion it must be said in passing that the name "hemolysis" is not limited to the biologic process just described; nor does it, as its etymology implies, necessarily indicate actual solution of the red cells. In practice the term is applied to any injury whereby their hemoglobin is liberated and diffuses through the surrounding fluid, and this may be effected by a variety of agencies, such as hypotonicity of the fluid, certain bacterial toxins, and mechanical damage such as results from prolonged agitation. With these forms of hemolysis we are not concerned in serologic work, except in so far as human serum received for the test, especially when sent from a distance, or guinea-pig serum obtained for use as complement, may sometimes be colored red by hemoglobin derived from corpuscles damaged by mechanical, bacterial, or other agencies. Serum so colored is unsatisfactory for use.

Application of the Principles of Bacteriolysis and Hemolysis.—It is necessary to bear constantly in mind the three substances or "bodies" which are concerned in bacteriolysis and in hemolysis and the part which each plays. This may be outlined as follows:

BACTERIOLYTIC SYSTEM

Antigen (invading bacterium)	+	Bacteriolytic amboceptor (in serum of infected person)	+	Complement (in serum of any normal animal)	=	Bacteriolysis

HEMOLYTIC SYSTEM

Antigen (red blood-corpuscles)	+	Hemolytic amboceptor (in serum of animal injected with red corpuscles)	+	Complement (same as in bacteriolytic system)	=	Hemolysis

The important fact in the above formulæ is that, while antigen and amboceptor differ in the two systems, the complement is the same. Whatever the source of the complement, it will serve either for bacteriolysis or for hemolysis, and *this is the key to the complement-fixation tests.*

In the application of these principles it is possible so to adjust the test that any two members of a system being known, the third may be determined qualitatively and (roughly) quantitatively. In the clinical use of the test, however, one seeks the amboceptor, whose presence in the patient's serum establishes the diagnosis of the corresponding disease. To accomplish this one mixes in a test-tube appropriate amounts of a culture or extract of the invading organism, blood-serum from a suspected patient, and complement. One of two things will occur:

(*a*) If the patient suffers from the disease in question and his blood-serum therefore contains the corresponding amboceptor, the complement will be fixed or bound to the antigen by this specific amboceptor, and no complement will be left in a free state.

(*b*) If the patient's serum does not contain the specific antibody to serve as a connecting link, the complement will remain unbound or free in the fluid.

In either case there will be no visible change to show what has taken place, and it is necessary to add an indicator which will show whether the complement still remains free. This is found in the two specific elements of the hemolytic system—red blood-corpuscles and hemolytic amboceptor. If free complement be pres-

ent the hemolytic system is completed and the corpuscles will be hemolyzed. If, upon the other hand, all available complement had been bound to the antigen by the antibody, then hemolysis cannot occur.

COMPLEMENT-FIXATION TEST FOR SYPHILIS

This is commonly called the Wassermann reaction, which is a convenient term, but not strictly correct, since Wassermann utilized principles already known. There is a tendency at present to restrict this name to the method originally employed by Wassermann and to slight modifications of it.

There are a number of well-recognized methods of performing the complement-fixation test for syphilis, with almost as many modifications as there are serologists. In general they fall into two divisions: those which follow Wassermann in using an anti-sheep hemolytic system and those which follow Noguchi in using an antihuman system. Each system has its merits and each its strong advocates. Anyone who can carry through one method with intelligence can easily learn any other.

One method of each class will be described in detail in the following pages. The antihuman method closely follows the standard technic adopted by the War Department and is practically Colonel Craig's modification of Noguchi's method. Owing to its relative simplicity it well serves as an introduction to the technic of complement-fixation tests, and it is therefore described in full detail and as simply as possible. It uses heated serum, amboceptor-serum dried on paper, two (preferably three) types of antigen, and primary incubation in the water-bath at 37° C. The antisheep method described is the new standardized quantitative method of Kolmer.[1] It employs one very sensitive antigen, with graduated amounts of serum, and primary incubation in the refrigerator. It is complicated and time consuming, but is given here because it represents the most careful attempt yet made to arrive at a standardized technic which shall be safeguarded against error at every point. It is a quantitative test, and appears to be more sensitive and at the same time to give fewer false positives than any method now in

[1] The technic of the test and the comparative studies which led to its adoption have been described in a series of thirty-two papers by Kolmer and his associates, published in the American Journal of Syphilis, 1919 to 1922.

general use. It is especially valuable as a check upon treatment. Whether the method can be simplified without loss of specificity remains for time to show.

A. Non-specific Nature of Test

In the preceding pages the complement-fixation test for syphilis is described as an application of the theory of bacteriolysis: as a test for the presence or absence of the specific antibody or bacteriolytic amboceptor which is antagonistic to *Treponema pallidum*. At the time when the test was devised *Treponema pallidum* had not yet been grown in pure culture, and pure suspensions of the organism were, therefore, not available as antigens. Wassermann obtained the organisms in as pure a state as was then possible by making extracts of syphilitic fetal liver, which contains these microorganisms in inconceivably large numbers; and such extracts proved to be satisfactory antigens. Later studies, after pure cultures of *Treponema pallidum* were available, developed the unexpected fact that pure suspensions of the organism do not serve at all well as antigens, and that the efficiency of extracts of syphilitic liver was not due to the many parasites present, but to certain lipoids or fat-like substances which are present in perfectly normal organs as well. At the present time alcoholic extracts of normal organs are almost universally used as "antigens," and the reaction can, therefore, no longer be regarded as due to the true antigen-antibody combination which the theory postulates. Nevertheless it is clear that there is present in syphilitic serum some peculiar substance, now called *reagin*, which, while it is doubtless not the true bacteriolytic amboceptor for *Treponema pallidum*, acts in an analogous way by binding complement to the lipoidal substances in the organ extracts. It is equally clear that this substance is highly distinctive of syphilis, being found in practically no other disease which might be confused with it.

The failure of the Wassermann reaction fully to accord with the theory upon which it was worked out does not necessarily diminish its dependability, but, on the contrary, serves to explain some of the fallacies and irregular results which have been encountered. Its practical value, in the hands of those who are able to interpret it, is unquestioned. It had, indeed, become well established before its lack of specificity was recognized, and it is probably fortunate

that pure cultures of *Treponema pallidum* were not available at the start, else they would have been used as antigen with disappointing results, and the test might have been promptly abandoned.

B. Laboratory Equipment for Complement-fixation Tests

1. **Apparatus Required.**—The following list is based upon the requirements of a small laboratory:

Animals.—It is necessary to keep four or more guinea-pigs to supply complement. They may be housed in suitable cages in the laboratory or, preferably, in a small well-ventilated room reserved

Fig. 287.—A high-power centrifuge with eight tubes suitable for serologic work. (Courtesy of International Equipment Co.)

for the purpose. The utmost cleanliness is necessary. They require a constant supply of clean water, and are fed vegetables and green stuff when obtainable, grains and dried fodder at other times. The animals do not stand cold well.

If the antisheep system is used, it is most satisfactory to keep a sheep as a source of the necessary corpuscles. Sheep corpuscles can, however, often be secured at an abattoir, or, in cities, from one of the large serologic laboratories. At times a sheep is found with resistant cells, so that there is trouble in obtaining satisfactory titrations with reagents that work satisfactorily with cells from other sheep.

Anesthetizing cone for guinea-pigs. Made of a mailing cylinder, with perforations in the bottom and side and absorbent cotton packed in the bottom. It should be large enough to admit the head of a guinea-pig.

Bandage, preferably of rubber, about 3 inches wide, for constricting the arm during puncture of a vein.

Centrifuge.—The ordinary physician's centrifuge with 15-c.c. tubes will answer if it runs smoothly; but a larger and more powerful instrument, which will also take 50-c.c. tubes, will be found more convenient (Fig. 287).

Centrifuge Tubes.—A dozen or more plain 15-c.c. tubes, four 50-c.c. tubes, and six graduated 15-c.c. tubes will probably be sufficient.

Glass tubing, soft, about 8 or 9 mm. outside diameter, for making capillary pipets and blood-capsules. The tubing must be of such size that standard rubber nipples fit snugly.

Hypodermic Syringes.—All-glass Luer syringes are recommended. The 10-c.c. size is most used. There should be a supply of needles of 19 and 21 gage. Platinum needles are preferable if expense does not prohibit.

Meat Grinder.—A small grinder for heart muscle to be used for making antigen.

Nipples (2 c.c.).—Heavy, "pure gum" nipples will give best service. Probably no more than five or six will be required.

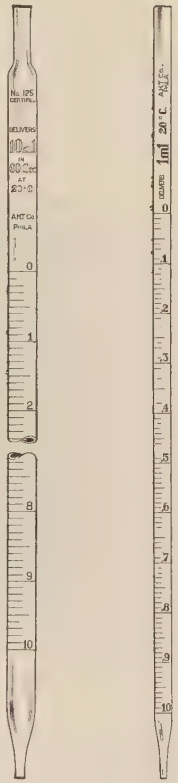


FIG. 288.—Serologic pipets: 10 c.c. graduated in tenths, and 1 c.c. graduated in hundredths. A desirable form, not graduated to the tip.

Pipets (Mohr's—Fig. 288).—The number will depend upon the amount of work to be done. As a start the following will probably be sufficient: three 10-c.c. pipets; eight 1-c.c. pipets, graduated in tenths; six 1-c.c. pipets, graduated in hundredths. They should be long and slender so that the graduations may be well separated.

Pipet box, cylindric, 2 x 16 inches, preferably of copper. Pipets may be sterilized and stored in this.

Refrigerator.—Any ice-box maintaining a temperature of 6° to 8° C. in the ice compartment will answer, but the new types of "iceless refrigerators," with electrically operated refrigerating plants installed, are very satisfactory.

Scales for weighing chemicals and balancing centrifuge tubes. Should be sensitive to 0.1 gm. The Harvard trip balance will be found satisfactory and reasonable in price.

Sterilizers, one for hot air, one for steam. Stew-pan for sterilizing syringes and small instruments (Fig. 289).

FIG. 289.—A very satisfactory pan for sterilizing small instruments by boiling, as suggested by Sanford. Holes are punched in the wall of an aluminum stew-pan above the water level. After sterilization the water is drained off through the holes as shown, without danger of pouring out the instruments.

Tags for guinea-pigs, numbered. These are fastened to the ears of the animals for purposes of identification.

Test-tubes.—The standard serologic tube is 100 x 12 mm., heavy wall, without flange. For Kolmer's method the *inside* diameter of all tubes must be uniform, 12 to 14 mm. Fifty to 100 of these will be required. A few smaller tubes, about 65 x 8 mm., will be found useful at times for holding small quantities of serum. These can be made from glass tubing when needed.

Test-tube Racks.—There must be at least one rack which will fit the water-bath. This is preferably of sheet copper, with a bottom platform and two perforated shelves which will hold the tubes without danger of their tipping. A convenient type has two rows

of ten holes each (Fig. 293). A rack with thirty-six holes (Fig. 294) will be found convenient for Kolmer's quantitative method. Another very convenient rack is made of heavy galvanized wire (Fig. 290). If a rice cooker serves as water-bath, a circular rack of a size to fit within it comfortably will be most satisfactory, and the top shelf may be made large enough to rest upon the rim of the opening so as to serve as a cover. A tin pan may then be inverted over this to assist in maintaining a uniform temperature. Two or three additional racks will be required to support tubes of serum and various reagents upon the work table. These may be made from a block of wood with auger holes of appropriate size to hold the necessary tubes.

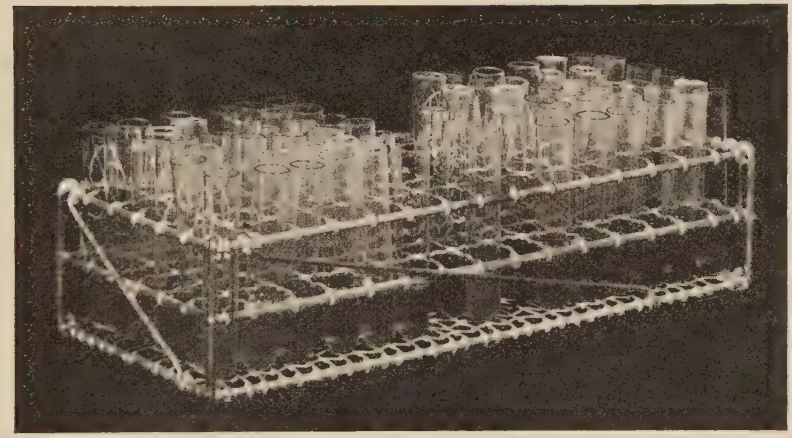


FIG. 290.—Galvanized iron wire rack for Kolmer-Wassermann tubes.

Water-bath.—A large double boiler or rice-cooker will answer admirably. A hole may be made in the lid for a cork with thermometer. With a little care the water in the inner compartment can be kept constantly at the same temperature by means of a small flame. When a large incubator is available a pan of water may be kept in this to serve as a water-bath. If expense does not prohibit, two electrically heated serologic baths—one to run at 56° C., one at 37° C.—will be found most satisfactory.

In addition to the above, much of the equipment in a clinical laboratory may be called into use as occasion requires.

2. **Care and Cleaning of Apparatus.**—The centrifuge must be oiled frequently, avoiding too much oil, which, if the instrument

be operated by electric power, will interfere with its running. It should never be run at higher speed than is necessary. Opposite tubes, with their contents, must be of the same weight, which is secured by standing them in tumblers on the pans of a balance and adding or removing fluid with a medicine-dropper.

Immediately after use hypodermic syringes should be thoroughly cleaned, sterilized if contaminated, and dried. Should blood coagulate within the needle the clot must be completely removed with a wire at once. The needle should then be thoroughly cleaned both inside and out with water, and dried by sucking through it alcohol, ether, and air in succession.

Hypodermic needles should be smooth outside and have sharp cutting points. The ability to pierce the skin easily and relatively painlessly is largely a matter of sharpness of the needle, which is appreciably dulled after one or two sterilizations. Needles may be sharpened by rubbing the bevel, point forward, on a fine carborundum hone. The outside of the needle is afterward polished by a few strokes of very fine emery paper such as jewelers use. Needles which have become clogged may be cleared with a jeweler's broach.

Graduated pipets should be placed in water immediately after use, or, if contaminated, in a cylinder of 5 per cent. *liquor cresolis compositus*. This is an excellent cleaning fluid as well as a disinfectant. When the day's work is done they should be thoroughly cleaned with water, using soap only when needed, rinsed repeatedly, drained dry, and placed in a metal box. When pipets are cloudy from the formation of a scum on the inner wall, they should be soaked for a time in bichromate cleaning fluid, and then rinsed thoroughly and repeatedly.

The test-tubes should be cleaned in the same way, using a test-tube brush, rinsed thoroughly and repeatedly with water, and drained dry. *The least trace of soap, alkali, or acid may interfere with the test*, hence it is often advised to avoid soap and chemicals altogether. The clean and dry tubes are packed, inverted, in wire baskets.

Capillary pipets are often thrown away after one use, but this is an unnecessary waste. Many of them will serve repeatedly. They are to be cleaned as described for graduated pipets, and dried by attaching a rubber nipple and sucking in and forcing out

alcohol, ether, and air successively Acetone may be substituted for the alcohol and ether. For baking and storage they may be placed in small tin boxes, such as are used for packing fancy cakes (Nabisco, and so forth).

It is highly desirable, but not absolutely essential, that all pipets and test-tubes be baked in a hot-air sterilizer at 180° C. for one-half hour.

C. Nature and Preparation of the Reagents

In making a complement-fixation test six essential "reagents" are employed: physiologic salt solution, antigen, complement, hemolytic amboceptor, red-cell suspension, and the fluid to be tested. The properties and preparation of all of these must be thoroughly understood, even though some of the reagents can be purchased ready prepared.

1. Physiologic Salt Solution.—This serves as a diluent. It is prepared by dissolving 8.5 gm. of chemically pure sodium chlorid in 1000 c.c. distilled water, and filtering. An impure sodium chlorid is unsuitable. In wet weather the salt should be dried in an oven before weighing. The solution should be placed in cotton-plugged test-tubes, flasks, or 4-ounce wide-mouth bottles, and sterilized in an Arnold sterilizer.

Mason and Sanford have emphasized the desirability of controlling the reaction of the salt solution by addition of buffer substances. They find that solutions of pure sodium chlorid in doubly distilled water are subject to so great variations in reaction, owing to absorption of carbon dioxid, as to disturb delicate titrations. The solution which they recommend consists of 7 gm. of sodium chlorid, 1.7 gm. of secondary sodium phosphate (2 molecules of water), and 0.2 gm. of primary potassium phosphate for each liter of doubly distilled water. This combination gives a solution with pH of from 7.4 to 7.8, and a salt concentration of 0.89 per cent.

2. The Syphilis Antigen.—(1) **Nature and Properties.**—As was explained in discussing the nature of the reaction, the function of the antigen as a reagent in the test is to serve as a base to which the *reagin* or syphilitic substance of the patient's serum can bind the complement; and the true antigen of syphilis, *Treponema pallidum*, serves much less efficiently in this rôle than do alcoholic extracts of normal organs (heart, liver, and so forth), either plain

or with cholesterol. These extracts are now used almost universally for the work, and are therefore called "syphilis antigens," although they are manifestly not true antigens in the biologic sense.

The one indispensable property of any antigen is its "antigenic power"—the power to unite with or "fix" complement in the presence of syphilitic serum. In addition to this, all antigens manifest to a greater or less degree two objectionable properties: all possess of themselves the power to absorb a certain amount of complement whether syphilitic serum be present or absent; and all, if used in sufficient quantity, are able of themselves to hemolyze red blood-corpuscles. These undesirable "anticomplementary" and "hemolytic" properties are independent of the antigenic power and are usually very much weaker than it is, so that they do not introduce any error into the test when a potent antigen is used in the customary amount. Information concerning these properties is obtained by three separate titrations, from the results of which one decides whether the particular antigen is fit for use, and, if satisfactory, what amount or "dose" should be used in the test.

To be satisfactory for use an antigen must be capable of binding sufficient complement in the presence of a strongly positive serum to cause complete inhibition of hemolysis when used in relatively small doses, for example, 0.02 to 0.1 c.c. of a 1 : 10 or 1 : 20 dilution. Upon the other hand, it must not of itself induce hemolysis or bind an appreciable amount of complement in the absence of syphilitic serum when used in amounts even five to ten times as great.

As to method of determining the dose which should be used in the test there is a lack of uniformity among laboratories. The precise amount is not so important as is the case with the other reagents. Any amount within certain well-defined limits will serve, and with a good antigen there is a fairly wide zone of choice. Many workers adopt one-fourth the largest amount which can be used without, of itself, binding enough complement to inhibit hemolysis. Others find the least amount which will give a completely positive reaction with a strongly positive syphilitic serum and use this or some multiple of it. The plans followed in Craig's and in Kolmer's methods are given later.

Some extracts remain good for many months, others more or less rapidly lose strength as antigens or develop anticomplementary

and hemolytic properties. For this reason each antigen must be carefully titrated at frequent intervals, *preferably every two weeks.*

It is well to preserve antigens in 10- or 15-c.c. bottles with tightly fitting glass stoppers. These are best kept in the ice-box. A slight sediment usually appears. This should not be disturbed, and care should be exercised not to take up any of it when pipeting off small quantities for titration or the test.

Before it can be used an antigen must be diluted with physiologic salt solution, a 1 : 10 dilution being generally most satisfactory for the ordinary antigen. Kolmer's antigen requires much greater dilution. The dilution must be freshly prepared each day, and the manner in which this is done is important. The amount of antigen likely to be used for the day's work, for example, 0.3 c.c., is placed in a test-tube and the requisite salt solution, 2.7 c.c., is added drop by drop with constant agitation. Another method is to gently layer the antigen upon the surface of the salt solution and allow the two to slowly mix by diffusion. The slow mixing produces a milky fluid, the maximum of turbidity being desirable because, for some unknown reason, the antigenic power is then at its highest level.

Since the various organ extracts which are used as antigens differ in their sensitiveness, it is customary as a routine procedure to test each suspected blood-serum with two or three antigens of different types, and the following may be recommended: (*a*) simple alcoholic extract of heart muscle; (*b*) cholesterolized extract; (*c*) Noguchi's acetone-insoluble extract. If only two are used, the second and third should be selected. Kolmer's method employs only his new cholesterolized and lecithinized antigen. This antigen may be used to advantage in any of the methods.

Antigens can be purchased ready prepared from a number of biologic supply houses. They are, however, not difficult to prepare if the following directions are carefully carried out.

(2) **Preparation of Antigens.**—(*a*) **Simple Alcoholic Extract of Heart Muscle.**—This antigen is not so sensitive as the two which follow, and sometimes gives a negative result when the others give a positive or partial positive.

Method of Neymann and Gager.—1. Obtain fresh normal human or beef hearts, remove fat, and mince strips of muscle finely in a meat grinder. Spread on glass plates and dry as quickly as possible with the

aid of an electric fan. Grind the resulting dried cakes into a fine powder, place in gauze bags, and dry for some days in an incubator. This powder may be kept indefinitely at room temperature in amber glass bottles. Mix the powder from several hearts.[1]

2. Place 25 to 50 gm. of the powder in a bottle or flask and cover with ether to a depth of about 2 inches. Keep at room temperature for from eight to ten days, shaking several times a day. During this period change the ether five or six times or until it is no longer colored yellow. Discard the used ether.

3. Dry the powder by spreading on glass or paper.

4. Place the powder in a wide-mouth bottle with enough 95 per cent. or absolute alcohol to cover it to a depth of $\frac{1}{2}$ inch. Place in an incubator for ten days, shaking several times a day.

5. Filter the alcohol and preserve for use as antigen.

6. Extract the powder a second time with alcohol in the same way for fourteen days and filter.

7. Both the first and the second alcoholic extracts should make good antigens. Titrate each for antigenic, hemolytic, and anticomplementary properties. Should the antigenic power not be sufficiently strong the extract may be evaporated to one-half or one-third its volume, although this will rarely be necessary.

(*b*) **Cholesterolized Extract of Normal Heart.**—This is also known as reinforced or fortified antigen. It is very sensitive, and for this reason has come into wide use. When syphilis is known to be present this antigen is excellent as a check upon the efficiency of treatment.

1. Prepare a simple alcoholic extract of heart muscle by the method of Neymann and Gager as above described.

2. To each 50 c.c. of the final filtrate add 0.1 to 0.2 gm. of Merck's cholesterol. Place in the ice-box for four or five days, shaking once or twice a day. It is best to prepare only a small quantity at a time, since the cholesterolized product does not keep well.

3. Filter through fat-free paper and titrate.

The following method is more rapid and less complicated and usually yields a good antigen:

1. Obtain a normal human or beef heart, remove fat, and cut out strips of muscle from the thickest part of the wall. Mince these finely in a meat grinder.

[1] This powder may be obtained of the Digestive Ferments Co., Detroit.

2. To each 10 gm. of the minced muscle add 100 c.c. absolute or 95 per cent. alcohol, and grind in a mortar.

3. Shake in a mechanical shaker for twenty-four to thirty-six hours. In the absence of a shaker place in the ice-box for ten days, shaking frequently during the day. Filter.

4. To this plain alcoholic extract add cholesterol in the proportion of 0.1 gm. for each 25 c.c. It is well to prepare only 25 c.c. at a time.

5. Place in the ice-box for ten days, shaking vigorously several times each day.

6. Filter and titrate.

(*c*) **Noguchi's Acetone-insoluble Extract.**—Preparation of this antigen is somewhat uncertain, but when a good extract is obtained it is sensitive and reliable and keeps well. Its anticomplementary power is usually very slight and does not require frequent titration.

1. Prepare a simple alcoholic extract of human or beef heart as described above.

2. Place about 500 c.c. of the final filtrate in a clean shallow dish at room temperature until the alcohol has completely evaporated. This may be hastened by use of an electric fan.

3. Dissolve as much as possible of the residue in about 200 c.c. of ether. Pour the turbid ether into a bottle and let stand overnight.

4. Pipet off the clear ether into a clean shallow dish, and evaporate it at room temperature to about one-fourth its volume.

5. To this concentrated ethereal solution add ten times its volume of pure acetone. A whitish precipitate should form.

6. Allow the precipitate to settle, pipet off as much as possible of the supernatant fluid, and allow the remainder to evaporate. A sticky yellowish mass is left.

7. Weigh the sticky residue, place in a bottle, and for each 0.3 gm. add 1 c.c. of ether and 9 c.c. of pure methyl alcohol. When it has dissolved the antigen is ready for use, provided that titration shows its properties to be satisfactory.

(*d*) **Kolmer's Cholesterolized and Lecithinized Antigen.**—This new antigen possesses exceptionally high antigenic power and very low anticomplementary and hemolytic powers. For use it must be diluted more highly than the antigens above described.

1. Prepare dried powder of normal beef hearts as described for the antigen of Neymann and Gager, page 595.

2. Place 25 gm. of the powder in a bottle with 200 c.c. of ether for five days, shaking several times each day. Remove the ether and preserve it.

3. Dry the extracted powder by spreading on a glass plate for several hours. Transfer to a bottle, and cover with 200 c.c. of 95 per cent. alcohol. Place in an incubator for four days, shaking occasionally.

4. Carefully pour off the alcohol into a flat dish, retaining the powdered muscle in the bottle for use in Step 6. Evaporate the alcohol under an electric fan and to the residue in the dish add 30 to 50 c.c. of ether. Stir well, cover, and let stand an hour or two for the insoluble particles to settle out. Pipet off the ether and add it to the ether used for the first extraction in Step 2 above.

5. Place the whole quantity of ether in a dish and evaporate to about one-fourth its volume, or 25 to 30 c.c. Now add six volumes (or about 150 c.c.) of pure acetone, stir well, and set aside, covered, overnight. A precipitate will form. Next day decant the acetone, transfer the sticky sediment to a bottle, cover with acetone, and preserve for use in Step 7.

6. To the muscle powder left after decanting the alcohol in Step 4 add 100 c.c. absolute, acetone-free ethyl alcohol. Cork tightly and place in an incubator for six days, shaking several times each day. If possible it should also be shaken for a day in a mechanical shaker. Filter this alcoholic extract through fat-free filter-paper and preserve for use in Step 7.

7. Dissolve 0.2 gm. pure cholesterol and all of the acetone-insoluble residue of Step 5 in 10 c.c. pure ether. Add this cloudy mixture to the filtered alcoholic extract of Step 6, and shake well.

8. Place the solution in the incubator overnight, and then keep at room temperature for a day or two, shaking occasionally, and finally filter through fat-free paper. This filtrate is the finished antigen and should be preserved in tightly stoppered brown glass bottles. Any precipitate which forms should be disregarded.

3. The Complement.—(1) **Nature and Properties.**—Unlike amboceptor, complement is not specific: the same complement will, in the presence of appropriate amboceptors, act upon various antigens, such as tubercle bacilli, gonococci, or red blood-corpuscles. It also differs from amboceptor in that it is relatively unstable. It deteriorates rapidly with age, the complement-containing serum

often becoming inert within a few days; and it can be completely destroyed by heating the serum to 56° C. for from fifteen to thirty minutes—a process which is known as "inactivation." Even during the incubation in the water-bath at 37° C., which is customary in complement-fixation tests, an appreciable amount of complement may be lost. For this reason water-bath incubation must not exceed two hours.

Within the past few years it has been shown that complement may be fixed by the antigen-reagin combination at ice-box temperature (1–10° C.). While such fixation is much slower than in the water-bath, requiring from twelve to eighteen hours, it appears to be more complete and more specific, and is employed with increasing frequency. It constitutes one of the features of Kolmer's method.

In order to understand the adjustment of the hemolytic system, which is an essential preliminary to every complement-fixation test, it is necessary to keep in mind certain important facts regarding the quantitative relations of complement, red corpuscles, and hemolytic amboceptor. The amounts of complement and amboceptor necessary to hemolyze a constant amount of red cells under the standard conditions of time and temperature are not absolutely fixed quantities, but, within certain limits, vary in inverse ratio. With fixed amounts of amboceptor and red-cell suspension, for example, a certain definite amount of complement is required to cause hemolysis. If, however, the amboceptor be increased, a less amount of complement will suffice to bring about the same result; or, if it be decreased, more complement will be required. It is thus a general rule that an excess of amboceptor in the test will compensate for a deficiency of complement, and vice versa; but this holds good only within certain limits. If either be reduced too far, hemolysis will not occur, no matter how great an excess of the other be used. If any change be made in the amount or strength of the red corpuscle suspension, it must be met by a corresponding change in either amboceptor or complement, or both. From these considerations it is clear that the hemolytic system may be adjusted by using appropriate amounts of any two of its three elements as constants, and finding the amount of the third which will just suffice to cause complete hemolysis. Many workers adopt standard amounts of red cells and complement and titrate to find the corre-

sponding amount of amboceptor. Others, including Craig, titrate the amboceptor, which is relatively stable, only once each month or two, and adjust the complement, which is variable in strength and unstable, on the day the tests are made. Kolmer titrates both complement and amboceptor each time.

(2) **Preparation of Complement.**—Complement is present in the blood-serum of practically all warm-blooded animals, but when derived from these different sources it differs considerably in its properties, chiefly in its complementary strength and in its fixability or power of forming a firm union with the antigen-amboceptor combination. In serologic work the guinea-pig has been almost universally adopted as the source of complement, since the serum is easily obtained and is satisfactory from all points of view. This serum is diluted and used in the test without further treatment.

Serum from different guinea-pigs often differs greatly in complementary activity, hence it is advisable to use mixed serum from two or three different animals. When possible the blood should be obtained the day before the tests are made. Serum appears to gain strength if it is allowed to stand upon the clot for twenty-four hours and to become less sensitive to the anticomplementary power of antigen and normal human serum.

Serum to be used as complement may be obtained by cutting the throat of a guinea-pig under anesthesia, collecting the blood in a suitable vessel, allowing it to clot, and pipeting off the serum. Care should be taken not to sever the esophagus, as contamination with gastric secretion would render the serum unfit for use.

This method is not, however, the method of choice. For the small laboratory the sacrifice of two or more guinea-pigs each time tests are run would be unduly wasteful. The following method of obtaining the blood by aspiration from the heart is therefore recommended:

1. Sterilize a 10-c.c. hypodermic syringe and No. 19 needle by boiling. Attach the needle and rinse out the syringe with physiologic salt solution.

2. Anesthetize a guinea-pig with ether. A cylindric "mailing case", with cotton in the bottom and a number of perforations in the end and sides, makes a satisfactory ether cone. It should be just large enough to easily admit the head of the animal. Some use a glass tumbler or a beaker.

3. Place the animal upon its back, swab the front of the thorax thoroughly with alcohol, and insert the needle through the chest wall directly into the heart (Fig. 291). A slight pull upon the plunger will cause the blood to rise in the syringe. Withdraw 3 to 7 c.c. of blood.

This procedure is easy after a little practice. With the animal upon its back, the needle is pushed vertically downward between the ribs close to the left edge of the sternum and about midway between the suprasternal notch and the tip of the ensiform cartilage at a point where the heart-beat can be felt with the finger. The needle should be sharp, otherwise it will pierce the tough skin with difficulty and may go through with a sudden plunge which will carry it entirely through the heart. To guard against this accident the wrist should be steadied upon the table beside the animal.

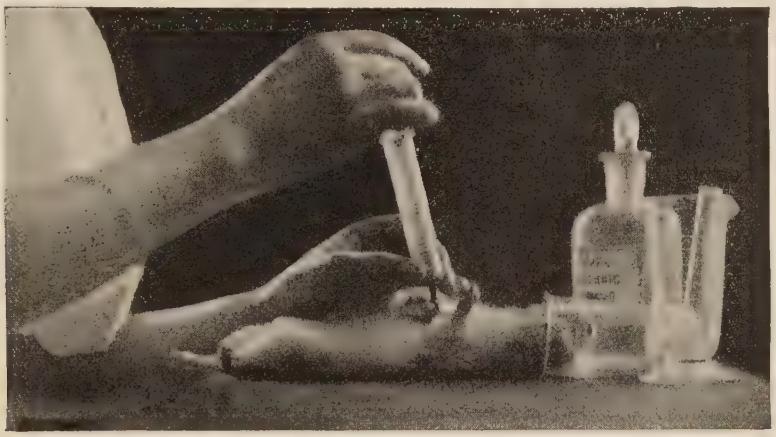


FIG. 291.—Showing method of securing blood from the heart of a guinea-pig (Thompson).

If the blood is not secured at the first or second trial, the guinea-pig should not be subjected to further punctures at that sitting. Rarely an animal will die during the operation or soon afterward, usually from the anesthetic, from injury to the bundle of His, or from hemorrhage; but ordinarily the animals suffer no injury to health if no more than from 5 to 10 c.c. of blood are taken every two weeks.

4. Discharge the contents of the syringe into a dry test-tube. Repeat the heart puncture with a second and even a third animal. A total of 10 c.c. of blood will usually suffice for the work of a small laboratory.

5. Let stand at room temperature until well coagulated. Gently loosen the clot from the wall of the tube and place in the ice-box for some hours, preferably overnight. If necessary, the serum may be separated at once by centrifugation.

6. By means of a capillary pipet with bulb transfer the serum to another tube, which, for the sake of convenience in diluting, may be a graduated centrifuge tube. If, in pipeting, the serum becomes cloudy from suspended red corpuscles, they should be thrown down with the centrifuge and the clear serum transferred to still another tube. It is then ready for dilution and titration as described later.

Complement-serum should be clear and of a light straw color. If it be red from hemolysis of corpuscles, as is likely to be the case when the syringe is not rinsed out with physiologic salt solution before the blood is drawn, it is unfit for use, but a pink tinge does no serious harm. A milky appearance is sometimes noted when the blood is taken after a full meal, and, if very marked, this also renders the serum unfit for use.

Ordinarily guinea-pig serum will remain active for three or four days if kept in a refrigerator. Various methods of prolonging this period of activity have been proposed, but none is entirely satisfactory. For use in his new method Kolmer recommends that 1.5 gm. chemically pure sodium chlorid be dissolved in each 5 c.c. of the guinea-pig serum. The serum is then kept at or near the freezing temperature in a brown glass bottle, and remains good for three weeks or longer. For use he adds 1 c.c. of serum to 29 c.c. of water, which gives the 1 : 30 dilution required for his test. Rhamy advocates the use of sodium acetate as preservative: When the guinea-pig serum is removed from the clot it is diluted with one and a half times its volume of 12 per cent. sodium acetate in physiologic salt solution, and is kept in the ice compartment of the refrigerator. Its complementary activity is preserved for weeks. Our own experience with this method has been slight, but satisfactory. Preserved complement, like fresh complement, must be titrated each time it is used, owing not only to deterioration of the complement but also to variations in the red cells.

4. The Hemolytic Amboceptor.—(1) **Nature and Properties.**—The hemolysin or hemolytic amboceptor used in the test is the antibody, which can be found in the blood-serum of a rabbit into which red blood-corpuscles from another species of animal have been injected until the rabbit's blood has acquired the power of destroying red corpuscles. The amboceptor is specific, that is, it will destroy no other red corpuscles than those of the particular species which caused its formation.

If red corpuscles be added to a serum containing the corresponding amboceptor, the amboceptor will unite with them, even at ice-box temperature, and the corpuscles are then said to be "sensitized." If, then, the red cells be centrifugalized out, the amboceptor will be removed with them. The union of complement with amboceptor is not so firm and does not take place so readily.

The activity of amboceptor-serum is not destroyed by heating for one-half hour at 56° C. (which destroys complement); and it remains active for a long time when preserved in a sterile condition. It may even be dried without great loss of strength and will keep in this condition for many months, even a year or two. Noguchi dried it upon filter-paper and used bits of the paper of predetermined size in his test. This plan is now widely followed for antihuman amboceptor and makes most decidedly for simplicity and convenience, especially in small laboratories. Antisheep hemolytic serum is almost universally kept in liquid form, preserved with an equal volume of glycerol.

While hemolytic amboceptor appears in the blood-serum in any considerable amount only as a result of the injection of the foreign red corpuscles, yet a small amount is sometimes naturally present. Of great practical interest is the presence of a variable amount of natural antisheep amboceptor in the blood of man. It has been shown that in more than 60 per cent. of human sera this is sufficient, without added amboceptor, to produce complete hemolysis of sheep corpuscles in the Wassermann test, and in less than 10 per cent. it is entirely absent. This is recognized as an important source of error in complement-fixation methods which employ an antisheep hemolytic system, since the unknown amount of amboceptor in the patient's serum is added to the accurately measured amount used in the test. To avoid this source of error Noguchi introduced the use of an antihuman hemolytic system. Some of those who use an antisheep system remove the variable amount of natural amboceptor by treating the human serum with washed sheep corpuscles. Upon the other hand, the Hecht-Weinberg-Gradwohl method utilizes this native amboceptor together with the native complement in the human serum instead of artificially prepared rabbit amboceptor and guinea-pig complement, first determining the amounts present by appropriate titrations. Antihuman hemolysin

dried on paper and antisheep hemolysin in ampules can be obtained from certain of the biologic supply houses.

(2) **Preparation of Hemolysin.**—Fully grown healthy rabbits are injected with washed red blood-corpuscles at frequent intervals. When it is thought that sufficiently strong hemolytic properties have been developed, a few drops of the rabbit's blood are tested. If it prove to be of satisfactory strength the rabbit is killed, its blood is collected and allowed to clot, and the serum is separated. Pieces of filter-paper are then impregnated with the serum and dried, or the serum is preserved in liquid form in ampules. Pure white rabbits with pink eyes appear to develop hemolysin more readily than do dark colored ones. Some rabbits will not respond satisfactorily to the injections, and an occasional one dies from anaphylaxis or other causes. It is, therefore, well to carry through several rabbits at the same time.

(*a*) **Preparation of Antihuman Hemolysin.**—This is much more difficult to prepare than is antisheep hemolysin; and, moreover, it is often highly agglutinative for human corpuscles, a property which may at times interfere with hemolysis in the test. The agglutinative power, however, diminishes in dried serum, and apparently much faster than does the hemolytic power.

By means of the blood-matching technic (p. 330) rabbits[1] should be selected whose serum does not naturally agglutinate human corpuscles, or, at most, only slightly. Fatalities among the rabbits are not infrequently due to agglutination of the injected corpuscles. Human corpuscles are toxic for rabbits, so that the animals become very thin during the course of the injections and may die from this cause.

1. **Preparation of Red Corpuscles.**—(*a*) Obtain blood from an arm vein of a healthy person in a sterile syringe which has been rinsed out with 1 per cent. sodium citrate in physiologic saline. The amount of blood will depend upon the number of rabbits and the plan followed for the injections.

At once remove the needle and empty the blood into a flask containing two or three times its volume of sterile 1 per cent. sodium citrate in physiologic salt solution, and mix.

[1] Guinea-pigs yield even more satisfactory amboceptor with less tendency to agglutination than do rabbits, and when only a small quantity is required it is preferable to use them. Five injections are made intraperitoneally at about four-day intervals, using 4, 6, 8, 12, and 15 c.c. of the 50 per cent. cell suspension.

In hospitals the red cells may be obtained by breaking up clots from the operating room in physiologic saline and removing particles of fibrin by filtering through cotton.

(*b*) Distribute among a number of sterile graduated centrifuge tubes and centrifugalize until the corpuscles have all gone to the bottom.

(*c*) Remove the supernatant fluid with a sterile capillary pipet with a large bulb.

(*d*) Mix the cells with sterile physiologic salt solution, and repeat the washings until the supernatant fluid is clear and colorless and fails to show any trace of albumin when tested by Heller's test. Four washings are usually sufficient.

(*e*) Add an amount of sterile physiologic salt solution equal to the volume of the corpuscles packed in the bottom of the tube and mix.

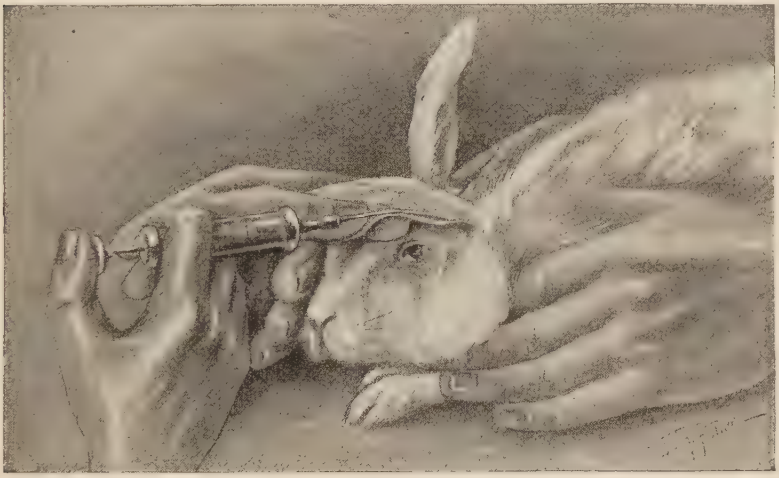


FIG. 292.—Showing method of injecting into the marginal vein of a rabbit's ear (Kolmer).

This makes a 50 per cent. suspension; that is, 2 c.c. contain 1 c.c. of corpuscles.

2. **The Injections.**—Portions of the 50 per cent. suspension are injected into the marginal ear veins of the selected rabbits with a hypodermic syringe and No. 21 needle (Fig. 292). The suspension need be only relatively sterile, as the rabbit's blood can easily take care of a few contaminating organisms. As to the amount used and the intervals between injections there is much divergence in the practice of different laboratories. The following two plans are among the best:

(*a*) *Army Medical School Method.*—Inject 6 c.c. of the 50 per cent. suspension daily for three days. Allow the rabbits to rest for twenty-one days, and then inject 1 c.c. of the 50 per cent. suspension daily for

five days. One week later draw a few cubic centimeters of blood and titrate for hemolytic power. If this is not strong enough, repeat the series of 1-c.c. injections.

(*b*) *Thompson's Method.*—Each day for three or four weeks inject into the marginal ear vein 0.2 c.c. of the above-described 50 per cent. suspension of washed human red corpuscles for each kilogram of the rabbit's weight. Since rabbits weigh about 2 kg., the usual amount injected is 0.4 c.c. of the suspension.

3. **The Preliminary Titration.**—(*a*) One week after the last injection withdraw 0.5 to 1 c.c. of blood from the rabbit's ear vein; or cut across a vein and receive blood in a small tube. Let coagulate; loosen the clot and centrifugalize; transfer the clear serum to a small test-tube and inactivate by heating in a water-bath at 56° C. for one-half hour.

(*b*) Make a 1 : 100 dilution by adding 1 part of serum to 99 parts of physiologic salt solution.

(*c*) Set up a series of seven tubes containing fixed amounts of salt solution, 5 per cent. suspension of human red cells (prepared as described on p. 609), and complement, and increasing amounts of the diluted rabbit's serum. The exact quantities are shown in the following table:

PRELIMINARY TITRATION OF HEMOLYTIC SERUM

Tube number.	Salt solution, 0.85 per cent.	Complement units.[1]	Red corpuscle suspension, 5 per cent.	Hemolytic serum diluted 1 : 100.
1.....	0.7 c.c.	2	0.1 c.c.	0.05 c.c.
2.....	0.7 c.c.	2	0.1 c.c.	0.10 c.c.
3.....	0.7 c.c.	2	0.1 c.c.	0.15 c.c.
4.....	0.7 c.c.	2	0.1 c.c.	0.20 c.c.
5.....	0.7 c.c.	2	0.1 c.c.	0.25 c.c.
6.....	0.7 c.c.	2	0.1 c.c.	0.30 c.c.
7.....	0.7 c.c.	2	0.1 c.c.	None.

(*d*) Incubate in water-bath at 37° C. for one hour. Make readings. Complete hemolysis in the first six tubes, or even in all of these but the first, corresponding to a "titer of 1 : 1000," will indicate that the serum is of satisfactory strength and the rabbit may be bled at once. The seventh tube is used to indicate whether the red cells and complement are right and should show no hemolysis whatever. Agglutination of the corpuscles sometimes makes trouble in this titration, but even then one can usually judge whether the serum has sufficient hemolytic power.

4. **Bleeding the Rabbit.**—(*a*) Anesthetize the rabbit; shave or clip

[1] If the unit of complement has not been determined by a previous titration assume 0.05 c.c. of a 1 : 1½ dilution in salt solution to be the unit.

the neck where the incision is to be made, and wash well with salt solution, but do not use a disinfectant.

(*b*) Make an incision to one side of the front of the neck, and cut the carotid artery. Avoid cutting the esophagus or trachea.

(*c*) Catch the blood in a large sterile glass dish, holding the rabbit over it in such a manner as to favor bleeding.

(*d*) Cover the dish and keep at room temperature for two hours, and then place in the ice-box overnight.

(*e*) Next day carefully transfer the clear serum to test-tubes by means of a capillary pipet with large bulb. The serum may show a tinge of pink, but more color than this is objectionable. If corpuscles are present they must be removed by centrifugation. Place the tubes in a water-bath at 56° C. for one-half hour.

5. **Impregnation of Paper.**—(*a*) Pour the serum into a deep Petri dish, which must be clean, dry, and sterile.

(*b*) Cut filter-paper into pieces of suitable size to fit the dish, allowing approximately 100 sq. cm. for each 1.5 c.c. of serum. Pieces 10 by 10 cm. are convenient to handle. The well-known S. and S. No. 597 paper is recommended.

(*c*) Immerse the sheets of paper in the serum one at a time. When they have become thoroughly soaked, lift out with forceps, drain against the edge of the dish to remove excess of serum, and place on a sheet of clean cheese-cloth. Dry as quickly as possible, preferably under an electric fan.

(*d*) Store the paper in amber glass bottles or other air-tight containers in a dark dry place.

(*b*) **Preparation of Antisheep Hemolysin.**—The method is analogous to that given above for antihuman hemolysin, but rabbits respond much more satisfactorily. The details need not be repeated.

Sheep corpuscles are secured as described on page 609, and washed about four times with saline. Kolmer advises five intravenous injections of 5 c.c. of 10 per cent. sheep-cell suspension, five days apart. Ten or twelve daily injections of 2 c.c. of 10 per cent. suspension will also serve.

One week after the last injection a small amount of blood is secured from the rabbit's ear, and allowed to coagulate. The serum is separated, transferred to a dry tube, and inactivated in a water-bath at 56° C. for fifteen minutes. The serum is then diluted 1 : 100, and the titration conducted as described on page 626.

If there is complete hemolysis in Tube No. 4, containing 0.5 c.c. of the 1 : 4000 dilution, the hemolytic power is satisfactory and the rabbit is bled as described on page 608. Should the titer not be sufficiently high the rabbit is given a few additional injections.

The antisheep hemolytic serum is diluted with an equal volume of sterilized pure glycerol, placed in vials or ampules, and stored in a refrigerator. The glycerin prevents bacterial growth, and in the high dilution used does not interfere in any way with the test. Kilduffe found such preserved serum to retain its activity practically unchanged for seven years.

5. Red Cell Suspension.—(1) **Properties.**—The red corpuscles of various animals, if used in conjunction with appropriate hemolysin, are capable of serving in complement-fixation tests. Their function is that of an indicator, giving visible evidence of the reaction which has taken place in the tube, but which could not otherwise be detected. Together with hemolysin and complement they make up the "hemolytic system." The classical Wassermann method employs sheep corpuscles; but, for reasons that are given under Nature of the Amboceptor, Noguchi introduced the use of human corpuscles.

For use the red corpuscles are washed and suspended in physiologic saline. The strength of the suspension and the amount to be used in the test are decided more or less arbitrarily, and the complement and amboceptor are adjusted to this. The adjustment, however, holds good only for the particular suspension employed in the titration. If another suspension be made, even though apparently of the same strength, a new titration is necessary, since corpuscles vary in their resistance to hemolysis.

The corpuscles settle quickly, hence, to insure absolutely uniform dosage, they must be thoroughly mixed each time any quantity is pipeted off. This is accomplished by repeatedly sucking the suspension into the pipet and forcing it out again.

While corpuscles not over twenty-four or forty-eight hours old are much to be preferred, they may still be used after three or four days in the ice-box, and they will often remain usable for two or three weeks if suspended in physiologic salt solution containing 1.25 c.c. formalin (40 per cent. formaldehyd) to the liter. In this solution the color changes to dark red. Old corpuscles must be washed anew before use; and they should not be used at all if the

second wash water be not free of hemoglobin, and if the normal red color do not return.

(2) **Preparation of Human Red Corpuscle Suspension.**—The corpuscles must be obtained from an individual belonging to Moss's iso-agglutination Group IV (p. 327) in order to avoid any possibility of agglutination by the human serum used in the test.

1. Prick the ear or finger deeply with a blood-lancet, and let 10 to 15 drops of blood fall into a graduated centrifuge tube containing 10 to 15 c.c. of physiologic saline, preferably with 1 per cent. of sodium citrate. Mix well after each drop. This is sufficient for about ten tests with the necessary titrations.

2. Wash three times as follows: (*a*) Centrifugalize until all corpuscles have gone to the bottom. This should not be too long nor at too high a speed, otherwise the corpuscles will become tightly packed in the bottom of the tube, and may be injured and hence rendered unduly susceptible to hemolysis.

(*b*) Remove the clear supernatant fluid with a capillary pipet.

(*c*) Fill the tube with physiologic salt solution, mix gently, and centrifugalize again.

3. After the third centrifugation, which should always be for the same length of time at the same speed, note the volume of the corpuscles packed in the bottom of the tube; make up to twenty times this volume with physiologic salt solution, and mix. If, for example, the corpuscles reached the 0.4 c.c. mark on the centrifuge tube, then add salt solution to the 8 c.c. mark. This gives the 5 per cent. suspension used in Craig's method.

(3) **Preparation of Sheep Corpuscle Suspension.**—The blood may be obtained at the abattoir, but it is much better to keep a sheep for the purpose. Small amounts of blood may be secured from an ear vein. Larger amounts are obtained from the external jugular vein as follows:

1. The sheep is backed into a corner and an assistant places himself astride its shoulders and holds its head up and back.

2. The wool is then clipped over the external jugular vein at the base of the neck and the area swabbed with alcohol.

3. With one hand the operator grasps the base of the sheep's neck just above the sternum, and makes pressure with the thumb over the vein, which will then stand out prominently.

4. With the other hand the operator plunges a sterile needle of No. 18 or larger gage into the vein, and lets the blood drip directly into a flask containing a 1 per cent. solution of sodium citrate in physiologic salt. No more than 1 part of blood to 4 parts of the solution should be taken. The pressure is then released and the needle withdrawn.

5. The citrated sheep's blood is washed as described above for human corpuscles. After the final washing, physiologic salt solution is added to make the 2 per cent. suspension used in Kolmer's method.

6. The Fluid to Be Tested.—Ordinarily the test is applied only to blood-serum, but upon occasion cerebrospinal fluid and other body fluids are examined.

(1) **Blood-serum.**—When necessary, the complement-fixation test can be performed with 0.3 c.c. of serum, which can be obtained from 15 to 20 drops of blood. This amount is easily secured in a Wright or a Lyon capsule from a puncture of the ear or finger-tip with a large (No. 2) straight Hagedorn needle or, better, a spring lancet.

As a rule, however, it is best to collect from 5 to 10 c.c. of blood. This allows the use of several antigens in the test, gives a reserve to fall back upon should anything go wrong, and, should the case turn out to be syphilitic, furnishes a supply for use as positive controls in subsequent tests. This amount of blood is secured by puncture of a vein as described on page 222. It must be placed at once in a dry sterile test-tube. Should the hypodermic syringe or the tube contain any water, sufficient hemolysis may occur to discolor the serum and possibly to disturb the test. Water left in a syringe after sterilization may be removed by rinsing with physiologic salt solution. If the blood is to be sent by mail, it should be placed in a small vial with a cork or rubber stopper. In order to minimize agitation through handling and thus lessen the likelihood of hemolysis the vial should be completely filled.

When the blood has coagulated firmly, the clot should be gently loosened from the glass by means of a needle and the tube stood in the ice-box for twelve to eighteen hours. By this time the serum will have separated nicely and is to be transferred to a clean tube by means of a capillary pipet. When there is need of separating the serum early it may be done by thorough centrifugation.

To be satisfactory for use serum must be clear, free from corpuscles, and not colored with hemoglobin. Should red corpuscles

be present, as is often the case when the serum is pipeted from the clot carelessly, they must be thrown down with the centrifuge and the clear serum again pipeted off into an appropriate tube. Because of their large diameter ordinary test-tubes make pipeting difficult when only a small amount of serum is available, and tubes about 8 mm. in diameter may be used. The serum may be lightly tinged with hemoglobin from injured corpuscles, but a strong red color renders it unfit for use. Serum which is opalescent or milky, as it may be when the blood is collected during the height of digestion, is sometimes, but not usually, anticomplementary.

Human serum contains a variable amount of complement, and, before the test is carried out, this must be destroyed by a process known as "inactivation"—heating in a water-bath at 56° C. for from fifteen to thirty minutes.

All serum absorbs or binds a certain amount of complement, but with fresh serum this absorption is usually slight and in no way interferes with the test, a small excess of complement being used in the test to offset it. Upon the other hand, old sera, especially when contaminated with bacteria, are prone to become so strongly anticomplementary that they will of themselves completely inhibit hemolysis. In order to insure recognition of such sera in the test, and thus to guard against the mistake of reporting a negative serum as positive, it is imperative to include a "serum control" tube for every serum tested. This tube contains the usual ingredients with omission of antigen; hence, if it show any inhibition of hemolysis, this must be caused by the complement-absorbing power of the serum itself irrespective of the existence of syphilis. Anticomplementary power may sometimes be removed by a second inactivation, but ordinarily a fresh specimen must be secured. In exceptional cases serum is uniformly anticomplementary even when fresh; and it may be necessary, as suggested by Breuer, to ascertain by a preliminary titration with different amounts of complement the exact quantity which the serum will absorb, and, when the test is set up, to add this amount of complement in excess of the amount usually used.

Sera known to be positive can be preserved for several weeks for use as controls in future tests by sealing in ampules and heating in a water-bath at 56° C. for one-half hour. This serves the double purpose of inactivating the complement and of killing off all bac-

teria unless spore-bearing forms chance to be present. Ruediger preserves positive serum by adding an equal quantity of sterilized pure glycerol. Since glycerol itself is anticomplementary, an amount equivalent to that introduced into the test with the serum should be used in all tubes of the complement titration.

(2) **Cerebrospinal Fluid.**—This is secured by lumbar puncture in the usual way. It must be clear. If red blood-corpuscles be present they should be centrifugalized out. The fluid is not to be inactivated. As a rule, several tubes are set up with different amounts of fluid, usually 0.2, 0.5, and 1 c.c., and the control tubes take like quantities.

Complement-fixation tests are sometimes applied to other body fluids, such as pleural and peritoneal transudates and exudates, milk, or albuminous urine. These are treated in the same way as spinal fluid. Fluids which contain pus, or which have become contaminated with bacteria, are likely to be strongly anticomplementary and hence unfit for the test.

D. Craig's Method—Antihuman Hemolytic System

This method is used in the laboratories of the United States Army. It is one of the least complicated of standard methods and is here given in full detail as an introduction to serologic technic.

1. Titration of Reagents.—Success in performing complement-fixation tests depends largely upon the use of proper reagents and the attainment of an exact balance between them. Employment of an unfit reagent or use of too large or too small a dose may completely reverse the results, changing a negative reaction into a positive, or vice versa. Even when prepared in the same way, reagents often differ in properties and strength, and they are, moreover, subject to deterioration. It is, therefore, necessary to test them carefully, both to assure one's self of their fitness for the work and to determine the amount or "dose" to be used. This testing is called titration, and is a time-consuming and important part of serologic technic.

The frequency with which the titrations must be repeated depends upon the stability of the particular reagent concerned. Complement, which is variable in strength and very unstable, must always be titrated upon the day on which it is to be used. Antigen and amboceptor-paper are more stable. The former re-

quires titration only every two or three weeks. The latter about once in two months. The red corpuscle suspension is made to standard strength by measurement and is then taken as a constant to which amboceptor and complement are adjusted.

It is self-evident that the titrations as well as the test itself must be set up with great accuracy; and no detail is more important than accurate pipeting. Facility and accuracy in the use of pipets comes only with practice. The end of the pipet and the finger must be dry and only the dry margins of the lips should be applied to the pipet. Any moisture will prevent exact control of the outflow of the liquid. The work of pipeting should be done where there is as little confusion as possible, and the worker should be seated to insure steadiness. In setting up a series of tubes, as for a titration, the pipet should be filled to the highest graduation and the fluid distributed among the tubes in regular order. The pipet is held vertically with its tip within the mouth of the tube and usually touching the inner wall, and the fluid is allowed to run in until the top of the fluid (represented by the bottom of the meniscus) falls to the desired graduation. The tip is then at once touched to the wall of the tube and quickly carried on to the next tube. It is necessary to make sure that the fluid runs down to the bottom of the tube, since, with very small quantities, as in some of the tubes of the complement titration, the fluid may remain clinging to the glass in the upper part and dry there. When there is only a small quantity of fluid, great care must be exercised in sucking it up, as the last portion is likely to go in with a rush and may reach the lips.

(1) **Titration of the Antigens.**—This must be repeated about once in three weeks.

1. The antigen is first carefully diluted 1 to 10 with physiologic salt solution, as described on page 595.

2. The least amount of this dilution which will bring about complete inhibition of hemolysis in the presence of 0.1 c.c., of a known strongly positive syphilitic serum, or, much better, 0.1 c.c. of a mixture of several positive sera, is then found by means of the following titration, the units of amboceptor and complement having been previously determined.

In the following titration Tube 7 is the serum-control and should give complete hemolysis, thus proving that the serum itself will not prevent hemolysis. Tubes 8 and 9 are controls of the hemolytic system. Tube 8

TITRATION OF ANTIGEN FOR ANTIGENIC POWER

Tube number.	Salt solution, 0.85 per cent.	Positive serum, inactivated.	Complement units.	Antigen emulsion, 1 : 10.	Shake. Incubate in water-bath at 37° C. for one-half hour.	Human red cells, 5 per cent.	Amboceptor paper units.	Incubate in water-bath at 37° C. for one hour. Shake every fifteen minutes. Place in ice-box for two hours or centrifugalize. Read results.
1.....	0.9 c.c.	0.1 c.c.	2	0.02 c.c.		0.1 c.c.	2	
2.....	0.9 c.c.	0.1 c.c.	2	0.03 c.c.		0.1 c.c.	2	
3.....	0.9 c.c.	0.1 c.c.	2	0.05 c.c.		0.1 c.c.	2	
4.....	0.9 c.c.	0.1 c.c.	2	0.10 c.c.		0.1 c.c.	2	
5.....	0.9 c.c.	0.1 c.c.	2	0.15 c.c.		0.1 c.c.	2	
6.....	0.9 c.c.	0.1 c.c.	2	0.20 c.c.		0.1 c.c.	2	
7.....	0.9 c.c.	0.1 c.c.	2	None		0.1 c.c.	2	
8.....	0.9 c.c.	None	2	None		0.1 c.c.	2	
9.....	0.9 c.c.	None	2	None		0.1 c.c.	None	

should show complete hemolysis; Tube 9 should show none. Tubes 1, 2, 3, 4, 5, and 6 show the antigenic power of the antigen: the amount in the first tube which shows complete inhibition of hemolysis is called 1 antigenic unit. Two to four times this amount, 2 to 4 antigenic units, may be used in the test. In practice it is often found that 0.1 c.c. of the 1 : 10 dilution falls within these prescribed limits; in such cases, for the sake of convenience in measurement, it is our custom to adopt 0.1 c.c. of this dilution as the dose to be used in the test. When the dose indicated by the titration falls much below this the dilution may be changed to bring the required amount in 0.1 c.c. A moderate excess of a good antigen does no serious harm, while too little may give negative results with such syphilitic sera as happen to be weaker than the one used in the antigenic titration. For this reason it is highly desirable to conduct the titration with pooled sera from several syphilitic persons. Should the antigen be so weak in antigenic power that the necessary dose exceeds 0.4 c.c. of a 1 : 10 dilution, it is unsatisfactory, since this amount makes the test fluid so cloudy that readings are difficult.

3. After the dose of antigen has been determined as above described, it is necessary to establish that an amount three or four times this dose will not possess appreciable anticomplementary power. The "set-up" for this titration is shown in the following table.

In the following titration Tube 6 is the serum-control and should show complete hemolysis, thus proving that the serum is not anticomplementary of itself. The last two tubes are controls of the hemolytic system; complete hemolysis should occur in Tube 7; none in Tube 8. Complete hemolysis in Tubes 1, 2, 3, and 4 indicates that the antigen fulfils the requirement that it be not anticomplementary in an amount equal

TITRATION OF ANTIGEN FOR ANTICOMPLEMENTARY POWER

Tube number.	Salt solution, 0.85 per cent.	Normal serum, inactivated.	Complement units.	Antigen emulsion.[1]	Shake. Incubate in water-bath at 37° C. for one-half hour.	Human red cells, 5 per cent.	Amboceptor units.	Incubate in water-bath at 37° C. for one hour. Shake every fifteen minutes. Place in ice-box for two hours or centrifugalize. Read results.
1.....	0.9 c.c.	0.1 c.c.	2	1 dose.		0.1 c.c.	2	
2.....	0.9 c.c.	0.1 c.c.	2	2 doses.		0.1 c.c.	2	
3.....	0.9 c.c.	0.1 c.c.	2	3 doses.		0.1 c.c.	2	
4.....	0.9 c.c.	0.1 c.c.	2	4 doses.		0.1 c.c.	2	
5.....	0.9 c.c.	0.1 c.c.	2	5 doses.		0.1 c.c.	2	
6.....	0.9 c.c.	0.1 c.c.	2	None.		0.1 c.c.	2	
7.....	0.9 c.c.	None.	2	None.		0.1 c.c.	2	
8.....	0.9 c.c.	None.	2	None.		0.1 c.c.	None.	

to four times the dose used in the test. With inhibition of hemolysis in Tube 4 the antigen may still be used, but with caution. Should Tube 3 show inhibition the antigen is unfit for use.

The use of normal serum in the anticomplementary titration is not absolutely necessary, but we prefer to include it because the conditions of the actual test are thus reproduced. Serum itself has some complement-binding power; it is, therefore, not the anticomplementary power of antigen alone, but of serum and antigen together, which may cause false positive reactions in the test.

4. The third titration of the antigen is undertaken to ascertain whether or not it possesses hemolytic power sufficient to introduce error into the test, and is carried out as indicated in the following table.

Incubate in water-bath at 37° C. for one hour. If the dose of antigen has not been previously determined, assume the dose to be 0.1 c.c. of a 1 : 10 dilution.

TITRATION OF ANTIGEN FOR HEMOLYTIC POWER

Tube number.	Salt solution, 0.85 per cent.	Complement units.	Antigen emulsion.	Red cell suspension, 5 per cent.
1.....	0.9 c.c.	2	1 dose.	0.1 c.c.
2.....	0.9 c.c.	2	2 doses.	0.1 c.c.
3.....	0.9 c.c.	2	3 doses.	0.1 c.c.
4.....	0.9 c.c.	2	4 doses.	0.1 c.c.
5.....	0.9 c.c.	2	5 doses.	0.1 c.c.
6.....	0.9 c.c.	2	None.	0.1 c.c.

[1] If the antigenic dose has not been previously determined, assume one dose to be 0.1 c.c. of a 1 : 10 dilution.

Hemolysis should not occur in any tube of the series. Tube 6 is a control and shows that the complement alone will not hemolyze red corpuscles. If there be any hemolysis, even slight, in Tubes 1, 2, 3, or 4, the antigen is not fit for use in the dose proposed.

(2) **Titration of Antihuman Amboceptor Paper.**—The preliminary titration of amboceptor-serum before it has been dried upon paper was described with the preparation of the amboceptor. There is distinct loss of strength in drying and the paper loses strength as time goes on, therefore the paper must be titrated before use and at intervals thereafter, usually about once in two months.

The edges of the paper, which are not likely to be uniformly impregnated with serum, are cut off and discarded. A strip of the paper of a convenient width, usually 3 to 5 mm., is then cut. Since the paper is rather brittle this may be done by scoring with a sharp needle along the edge of a ruler; or the paper may be ruled with a hard lead pencil and cut with scissors. More satisfactory is the use of the "amboceptor cutter" devised by Colonel Vedder. This consists of a small trimming board, such as is used for photographic prints, fitted with an adjustable gage which makes it possible to cut strips of the desired width easily and accurately.

The strips of paper are then cut crosswise into small pieces of different widths, usually 1, 2, 3, 4, and 5 mm., and those are used in the titration, which is set up as indicated in the following table. The small pieces are handled with forceps or by means of a needle upon which they are impaled.

TITRATION OF AMBOCEPTOR PAPER

Tube number.	Salt solution, 0.85 per cent.	Complement units.[1]	Red cell suspension, 5 per cent.	Amboceptor paper.
1.....	0.9 c.c.	2	0.1 c.c.	5 by 1 mm.
2.....	0.9 c.c.	2	0.1 c.c.	5 by 2 mm.
3.....	0.9 c.c.	2	0.1 c.c.	5 by 3 mm.
4.....	0.9 c.c.	2	0.1 c.c.	5 by 4 mm.
5.....	0.9 c.c.	2	0.1 c.c.	5 by 5 mm.
6.....	0.9 c.c.	2	0.1 c.c.	None.

[1] If the unit of complement has not been previously determined by titration against a known amboceptor, assume it to be 0.05 c.c. of 1 to 1½ dilution of pooled serum from three or more guinea-pigs.

Incubate in water-bath at 37° C. for one hour, and read results. The first tube which shows complete hemolysis, being perfectly clear with no trace of cloudiness, contains one unit of amboceptor. Twice this amount, or two units, is used in the various titrations and in the test proper. If, for example, complete hemolysis were effected by a piece 5 by 5 mm. in size, the double unit used in the test would be 5 by 10 mm. As a matter of fact, good amboceptor paper will usually be stronger than this, often allowing a double unit as small as 5 by 4 mm.

(3) **Titration of Complement.**—The titration of the complement is an essential preliminary to the test and must be done upon the same day.

The method of securing complement serum has been described in a previous section. Before use the serum is diluted with physiologic salt solution. The exact dilution is of no great consequence. but a 40 per cent. dilution, made by mixing the serum with one and a half times its volume of salt solution, is generally satisfactory. Care must be taken that the serum and saline are well mixed. The titration is then set up as follows:

TITRATION OF THE COMPLEMENT

Tube number.	Salt solution, 0.85 per cent.	Human red cells, 5 per cent.	Complement serum, diluted.	Amboceptor paper, units.
1.....	0.9 c.c.	0.1 c.c.	0.02 c.c.	2
2.....	0.9 c.c.	0.1 c.c.	0.03 c.c.	2
3.....	0.9 c.c.	0.1 c.c.	0.04 c.c.	2
4.....	0.9 c.c.	0.1 c.c.	0.05 c.c.	2
5.....	0.9 c.c.	0.1 c.c.	0.06 c.c.	2
6.....	0.9 c.c.	0.1 c.c.	0.07 c.c.	2
7.....	0.9 c.c.	0.1 c.c.	0.08 c.c.	2
8.....	0.9 c.c.	0.1 c.c.	0.09 c.c.	2
9.....	0.9 c.c.	0.1 c.c.	0.10 c.c.	2
10.....	0.9 c.c.	0.1 c.c.	0.10 c.c.	None.

In order to avoid sensitization of the red cells add reagents in the order given. Incubate in water-bath at 37° C. for one hour.

Read results at once, as hemolysis continues after incubation has ceased. The first tube that shows complete hemolysis, the fluid being

perfectly clear with *no trace whatsoever of cloudiness*, contains 1 unit of complement. In practice, this most often falls between the second and sixth tubes. *Two units*, or twice the amount necessary to cause hemolysis, are used in the test. The reason for using the excess of complement is found in the fact that both antigen and normal human blood-serum absorb a certain amount, and this would not leave enough to cause hemolysis if only the exact unit of complement were used in the test. One extra unit is arbitrarily selected as sufficient to cover this non-specific absorption, but not sufficient to bring about a negative reaction in any case which would otherwise be positive. It would, of course, be possible to find by titration the exact amount of complement absorbed by the antigen and by the particular serum tested, and to add just this amount in excess of one unit; but this would introduce complexities into the test, and is not found necessary in practice.

2. The Test Proper.—For one who is familiar with the properties of the various reagents and who has mastered the technic of the titrations, the test itself will offer few difficulties. It will then amount to little more than the carrying out of a long series of relatively simple procedures in an orderly manner. Accuracy in the work will, however, depend largely upon the adoption of a definite routine and strict adherence to it.

Preparations should be started the day before the test. Make sure that a sufficient number of clean, dry, baked tubes and pipets are on hand (for preparation see p. 592); also a supply of sterile physiologic salt solution. Secure the patient's blood, a known negative blood, and a known positive blood (unless positive serum be already on hand); label carefully, and give an identification number. Obtain blood for complement from two or more guinea-pigs. Secure red corpuscles from an individual belonging to Moss' isohemagglutination Group IV and wash three times (p. 609).

When ready to begin work on the day of the test arrange all articles which are likely to be needed upon the table within easy reach. To avoid confusion, each item should have its regular place. The table should be of such height that the worker may be seated while setting up the test.

1. Centrifugalize the guinea-pig blood obtained the day before. With a capillary pipet transfer the clear serum to a graduated centrifuge tube. Dilute with physiologic salt solution to two and a half times its volume,

thus making a 40 per cent. dilution for use as complement. Mix thoroughly and stand in reagent rack on work table.

2. Cut a sufficient number of pieces of amboceptor paper, 2-unit size.

3. Take the red-cell suspension from the ice-box, wash once if there be any trace of hemolysis, centrifugalize for the standard length of time, make up to twenty times the volume of the cells with salt solution, mix well, and place in reagent rack on work table.

4. Set up the complement titration. Incubate for one hour, removing rack and shaking well every fifteen minutes. Use care that none of the pieces of amboceptor-paper remain clinging to the wall of the tubes above the level of the fluid.

5. While the complement titration is incubating centrifugalize the blood to be tested, transfer the clear serum to appropriate tubes by means of a capillary pipet, using a fresh pipet for each serum, and with a wax pencil mark each tube clearly with its identification number. Inactivate the serum by placing the tubes in the water-bath at 56° C. for one-half hour.

6. When the complement-incubation is finished, remove rack and make reading in a good light. The tube accepted as showing complete hemolysis must be crystal clear. Record the amount of complement-serum in this tube as the unit of complement.

7. Take the stock antigens from the ice-box. With a 1-c.c. pipet graduated in tenths, remove from each the amount likely to be needed for the day's tests, usually about 0.2 c.c. for the work of a small laboratory. Dilute each in a test-tube with nine times its volume of salt solution as described on page 595. Mark each tube of diluted antigen with appropriate letters (for example, "C. H." for cholesterolized extract of heart), and place in the reagent rack on the following work table.

8. Set up the test as indicated in table and in Figure 293 (p. 620), using at least two and preferably three types of antigen: plain extract of heart muscle, cholesterolized extract, and acetone-insoluble extract. Kolmer's antigen may also be used with advantage. Mark all tubes clearly with a wax pencil, indicating the sera by their identification numbers, and the antigens by their initials. Add the fluids in the order given, first placing salt solution in all tubes, then the sera in all tubes, and so forth. Use a separate pipet for each serum. For the salt solution use a 10-c.c. pipet, graduated in tenths, and place a wax-pencil mark opposite each ninth graduation.

9. If the antigens have not been titrated within two weeks, it is necessary to set up an anticomplementary and a hemolytic control for each antigen. For the anticomplementary control Tube 2 of the anticomplementary titration (p. 614) is prepared for each antigen and

"SET UP" FOR CRAIG'S METHOD

USING TWO ANTIGENS: CHOLESTEROLIZED HEART, "C. H.," AND ACETONE INSOLUBLE, "A. I.," WITH POSITIVE AND NEGATIVE CONTROLS, AND ONE UNKNOWN SERUM

	Tube number.	Place in rack.	Salt solution, c.c.	Inactivated serum.	Complement, units.	Antigen emulsion.	Human red cells, 5 per cent. c.c.	Amboceptor paper units.
Negative Control.	1	First hole front.	0.9	Normal serum, 0.1 c.c.	2	Antigen "C.H." 1 dose.	0.1	2
	2	Second hole front.	0.9	Normal serum, 0.1 c.c.	2	Antigen "A.I." 1 dose.	0.1	2
	3	Second hole back.	0.9	Normal serum, 0.1 c.c.	2	None.	0.1	2
Positive Control	4	Third hole front.	0.9	Syphilitic serum, 0.1 c.c.	2	Antigen "C. H." 1 dose.	0.1	2
	5	Fourth hole front.	0.9	Syphilitic serum, 0.1 c.c.	2	Antigen "A.I." 1 dose.	0.1	2
	6	Fourth hole back.	0.9	Syphilitic serum, 0.1 c.c.	2	None.	0.1	2
Unknown	7	Fifth hole front.	0.9	Unknown serum, 0.1 c.c.	2	Antigen "C. H." 1 dose.	0.1	2
	8	Sixth hole front.	0.9	Unknown serum, 0.1 c.c.	2	Antigen "A.I." 1 dose.	0.1	2
	9	Sixth hole back.	0.9	Unknown serum, 0.1 c.c.	2	None.	0.1	2

Mix well. Incubate in water-bath at 37° C. for one-half hour. Remove and add red cells and amboceptor-paper.

Incubate in water-bath at 37° C. for one hour. Shake every fifteen minutes. Place in ice-box for two hours, or centrifugalize, and read results.

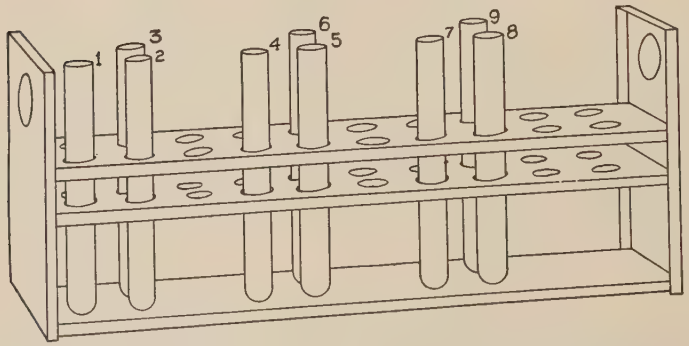


FIG. 293.—Rack with tubes showing "set-up" for Craig's method. The tubes are numbered to correspond with the chart.

placed in the rack with the test. The hemolytic control tubes are set up like Tube 3 of the hemolytic titration of the antigen (p. 615), and are placed in the rack at the end of the half-hour incubation period.

10. When the test is set up, place in the water-bath at 37° C. and record the time. The incubation period is one-half hour.

11. At the end of this time remove the rack and add the red cell suspension and amboceptor paper to each tube. Make sure that the red cells are thoroughly mixed immediately before each pipetful is removed. Replace for one hour's incubation. Record the time. Remove the rack every fifteen minutes and shake well. Make sure that none of the pieces of amboceptor paper remain clinging to the wall of the tubes above the level of the fluid.

12. After an hour's incubation remove the rack and make a preliminary reading. Place in the ice-box for two hours or overnight and make final reading; or centrifugalize all the tubes and make the final reading at once.

3. Reading and Recording Results.—Readings are frequently made by experienced workers immediately after removing the racks from the water-bath, but it is strongly recommended that final readings be not made until the tubes have been centrifugalized, or until they have stood in the ice-box for two hours. When all unhemolyzed corpuscles have gone to the bottom readings are made with ease.

Tubes in which hemolysis has been completely inhibited will have a heavy red sediment of corpuscles, and the supernatant fluid will show no trace of red whatsoever.

Partial hemolysis is evidenced by a reddish coloration of the fluid, varying, according to the degree of hemolysis, from a barely perceptible tinge to a strong red, and accompanied by progressively lessened amount of the red sediment. With experience it will be possible to estimate the degree of hemolysis from the depth of color of the fluid, but at first it is well to compare this color with that of hemoglobin solutions of known strength. Such standard solutions can be made from the normal control tubes, which show complete hemolysis, as follows:

To produce the color of 25 per cent. hemolysis take 0.25 c.c. of fluid from the hemolyzed tube and add 0.75 c.c. salt solution and the dose of antigen. To produce the color of 50 per cent. hemolysis

take 0.5 c.c. from the hemolyzed tube and add 0.5 c.c. salt solution and the dose of antigen.

In tubes showing complete hemolysis the fluid will be red, and there will be no trace of red sediment: it will be crystal clear, or, if antigen be present, somewhat cloudy, since antigen itself introduces a degree of turbidity. Not infrequently there may be in those tubes a slight sediment, consisting of the stroma of red cells which have given up their hemoglobin but which have not yet been completely dissolved. In such cases the sediment is whitish, not red, and must not be interpreted as indicating inhibition of hemolysis.

In inspecting the tubes it is well to adopt a definite routine as follows:

(*a*) *Inspect the serum controls in the back row, Tubes 3, 6, 9.* All should show complete hemolysis. Should there be any inhibition in any tube, shown by a deposit of red cells upon standing, the serum is anticomplementary. If this be marked, the results in the corresponding front-row tubes cannot be trusted. If, upon the other hand, the anticomplementary effect is slight, it may be possible to overcome it by incubating until the serum-control tube is completely hemolyzed; but it must be recognized that even in the case of negative sera inhibition if present in the control tubes will ordinarily be greater in the corresponding front-row tubes owing to the presence of antigen, which also absorbs a certain amount of complement.

(*b*) *Read the known negative control, Tubes 1 and 2.* These should show complete hemolysis, although, owing to the presence of antigen, the fluid may not be crystal clear. Should there be any inhibition something is wrong. The exact cause of the trouble may be difficult to determine: it may be that the antigen is anticomplementary in the dose used, that there was an error in pipeting, that the amboceptor-paper clung to the wall of the tube above the fluid, or that the unit of complement adopted was too small.

(*c*) *Read the known positive control, Tubes 4 and 5.* There should be complete inhibition of hemolysis. If the fluid shows any reddish tinge after the corpuscles have settled, either the antigen lacks sufficient antigenic power in the amount used; or it is hemolytic of itself; or too much complement was used through an error in pipeting or through adoption of too large a unit.

(*d*) *Read the unknown serum, Tubes 7 and 8.* These are read only after inspection of all controls have proved the reagents and conditions to be right. They may show complete hemolysis, indicating a negative reaction; complete inhibition, indicating a fully positive reaction; or any degree of hemolysis between the two extremes. The following scheme for recording results is in general use:

Complete, or *almost complete, hemolysis*, exceeding 75 per cent. of the corpuscles, indicates a negative reaction, recorded as "negative" "−." When hemolysis is not quite complete the expression "plus-minus" is often used, but this additional term is not necessary.

Partial hemolysis, between 50 and 75 per cent. of the corpuscles, indicates a doubtful reaction, recorded as "one plus" or "+."

Partial hemolysis, between 25 and 50 per cent. of the corpuscles, indicates a very weakly positive reaction, recorded as "two plus" or "++."

Slight hemolysis, not exceeding 25 per cent. of the corpuscles, indicates a weakly positive reaction, recorded as "three plus" or "+++."

Complete inhibition of hemolysis indicates a strongly positive reaction, recorded as "four plus" or "++++."

In reporting to persons who are not accustomed to the above plan it is well to report a "four plus" reaction as "strongly positive," a "three plus" as "questionably positive," a "two plus" as "doubtful," and a "one plus" or less as "negative."

4. The Test with Fluids Other than Blood-serum.—The technic of the test is slightly modified for spinal fluid, or transudates. If cloudy, these fluids are clarified as much as possible by centrifugation. They should not be inactivated as is blood-serum. For the test three tubes, containing respectively 0.2, 0.5, and 1 c.c. of the fluid, are set up for each antigen, and controls containing corresponding amounts without antigen are placed behind them in the rack. Fluids which contain pus or which have become contaminated with bacteria are likely to be strongly anticomplementary.

5. The Quantitative Method.—As ordinarily performed Craig's test gives no index of the amount of complement binding substance, or reagin, in the patient's serum except when the reaction is less than four plus. Probably the best way to secure quantitative results is to set up the test with a series of tubes containing decreasing amounts of the patient's serum—0.1, 0.05,

0.02, 0.01 c.c., and so forth—until a quantity is found which just fails to give a four-plus reaction.

E. Kolmer's Method—Antisheep Hemolytic System[1]

1. The Titrations.—(1) **Titration of Antigen.**—Only one antigen is used. This is Kolmer's cholesterolized and lecithinized alcoholic extract, the preparation of which is described on page 597. The dose to be used in the test is ten times the antigenic unit, and this amount must be at least twenty times less than the anticomplementary or hemolytic unit. The volume used in the titrations is always 0.5 c.c.; it is the dilution which is varied in the different tubes.

1. For the **hemolytic titration** prepare a series of dilutions with physiologic salt solution as follows:

1.0 c.c. antigen		+ 3.0 c.c. saline	= 1 : 4	dilution
0.5 c.c. antigen		+ 2.0 c.c. saline	= 1 : 5	dilution
0.5 c.c. antigen		+ 2.5 c.c. saline	= 1 : 6	dilution
1.0 c.c. of 1 : 4	dilution	+ 1.0 c.c. saline	= 1 : 8	dilution
1.0 c.c. of 1 : 5	dilution	+ 1.0 c.c. saline	= 1 : 10	dilution
1.0 c.c. of 1 : 6	dilution	+ 1.0 c.c. saline	= 1 : 12	dilution
1.0 c.c. of 1 : 8	dilution	+ 1.0 c.c. saline	= 1 : 16	dilution
1.0 c.c. of 1 : 10	dilution	+ 1.0 c.c. saline	= 1 : 20	dilution
1.0 c.c. of 1 : 12	dilution	+ 1.0 c.c. saline	= 1 : 24	dilution
1.0 c.c. of 1 : 16	dilution	+ 1.0 c.c. saline	= 1 : 32	dilution

Using dilutions as shown above set up a series of tubes as shown in first table, page 625. Note that each tube contains 0.5 c.c. of one of the dilutions.

The tube containing the highest dilution which produces any hemolysis indicates the hemolytic unit.

2. For the **anticomplementary titration** of the antigen use the same series of dilutions and set up tubes as shown in second table on page 625.

With a good antigen the majority of the tubes will show complete hemolysis. The tube containing the highest dilution which causes any

[1] By slight modifications, chiefly a change in the dilution of complement serum, this method may be used with antihuman, antiox, or antichicken hemolytic systems. The quantitative test is slightly less sensitive with the antihuman and antichicken systems, while with the qualitative test results are almost identical. For details see Kolmer, J. A.: The New Complement-fixation Test for Syphilis Conducted with Antihuman, Antichicken, and Antiox Hemolytic Systems, Amer. Jour. Syph., vol. 6, p. 667, October, 1922.

TITRATION OF KOLMER'S ANTIGEN FOR HEMOLYTIC PROPERTIES

Tube number.	Antigen dilution 0.5 c.c.	Salt solution, 0.85 per cent.		Sheep corpuscles, 2 per cent.	
1	1 : 4	2 c.c.	Mix well and place in refrigerator at 6° to 8° C. for fifteen to eighteen hours.	0.5 c.c.	Place in water-bath at 38° C. for one hour. Transfer to refrigerator for several hours and read results.
2	1 : 5	2 c.c.		0.5 c.c.	
3	1 : 6	2 c.c.		0.5 c.c.	
4	1 : 8	2 c.c.		0.5 c.c.	
5	1 : 10	2 c.c.		0.5 c.c.	
6	1 : 12	2 c.c.		0.5 c.c.	
7	1 : 16	2 c.c.		0.5 c.c.	
8	1 : 20	2 c.c.		0.5 c.c.	
9	1 : 24	2 c.c.		0.5 c.c.	
10	1 : 32	2 c.c.		0.5 c.c.	

TITRATION OF KOLMER'S ANTIGEN FOR ANTICOMPLEMENTARY PROPERTIES

Tube number.	Antigen dilution 0.5 c.c. or saline.	Inactivated normal human serum, 1 : 10.	Complement, 2 full units.[1]		Antisheep amboceptor (2 units).	Sheep's corpuscles, 2 per cent.	
1	1 : 4	0.5 c.c.	1.0 c.c.	Mix well. Place in refrigerator at 6° to 8° C. for fifteen to eighteen hours, followed by water-bath at 38° C. for five minutes.	0.5 c.c.	0.5 c.c.	Mix. Place in the water-bath at 38° C. for one hour. Transfer to refrigerator for a few hours and read results.
2	1 : 5	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	
3	1 : 6	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	
4	1 : 8	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	
5	1 : 10	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	
6	1 : 12	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	
7	1 : 16	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	
8	1 : 20	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	
9	1 : 24	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	
10	1 : 32	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	
11	0.5 saline	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	
12	0.1 saline	None	1.0 c.c.		0.5 c.c.	0.5 c.c.	

[1] For method of determining the "full unit" of complement see page 629.

inhibition of hemolysis indicates the anticomplementary unit. Tube 11 is the serum-control and Tube 12 the control for the hemolytic system. Both should show complete hemolysis.

3. For the **antigenic titration** much higher dilutions are necessary. Using the remainder of the 1 : 10 dilution originally prepared, make a further series of dilutions in ten test-tubes as follows:

0.1 c.c. of 1 : 10	antigen dilution	+ 2.9 c.c. saline	= 1 : 300	dilution
0.1 c.c. of 1 : 10	antigen dilution	+ 3.9 c.c. saline	= 1 : 400	dilution
0.1 c.c. of 1 : 10	antigen dilution	+ 4.9 c.c. saline	= 1 : 500	dilution
1.0 c.c. of 1 : 300	antigen dilution	+ 1.0 c.c. saline	= 1 : 600	dilution
1.0 c.c. of 1 : 400	antigen dilution	+ 1.0 c.c. saline	= 1 : 800	dilution
1.0 c.c. of 1 : 500	antigen dilution	+ 1.0 c.c. saline	= 1 : 1000	dilution
1.0 c.c. of 1 : 600	antigen dilution	+ 1.0 c.c. saline	= 1 : 1200	dilution
1.0 c.c. of 1 : 800	antigen dilution	+ 1.0 c.c. saline	= 1 : 1600	dilution
1.0 c.c. of 1 : 1000	antigen dilution	+ 1.0 c.c. saline	= 1 : 2000	dilution
1.0 c.c. of 1 : 1200	antigen dilution	+ 1.0 c.c. saline	= 1 : 2400	dilution

Arrange the antigenic titration as shown in the table on page 627. The syphilitic serum should be a mixture of equal parts of four or more strongly positive sera which have been inactivated at 55° C. for at least fifteen minutes. For use the serum is diluted by adding nine times its volume of salt solution and mixing well.

Tubes 11 and 12 (table, p. 627) are controls of the serum and the hemolytic system respectively, and should show complete hemolysis. Of the other tubes some will show complete inhibition, others incomplete inhibition, or none. The highest dilution which causes complete inhibition is the antigenic unit. The dose used in the test is 10 antigenic units, and these are contained in 0.5 c.c. of a dilution ten times as strong as that which gives the unit. If, for example, the highest dilution which caused complete inhibition was 1 : 2000 (Tube 9), the amount to be used in the test is 0.5 c.c. of a 1 : 200 dilution. It is imperative, however, that this amount be at least twenty times less than the anticomplementary or hemolytic unit.

(2) **Titration of Antisheep Amboceptor for Kolmer's Method.**—This method requires that both amboceptor and complement be titrated each time tests are run, using the same complement and red corpuscles to be employed in the test. The amboceptor-serum is kept in liquid form, preserved with an equal volume of glycerol. The volume used in titrations and in the test is always 0.5 c.c., and it is the purpose of the titration to find how greatly the amboceptor serum must be diluted to allow of this dose.

TITRATION OF KOLMER'S ANTIGEN FOR ANTIGENIC POWER

Tube number.	Antigen dilution, 0.5 c.c. or saline.	Inactivated syphilitic serum, 1 : 10.	Complement, (2 full units).	Mix well. Incubate in refrigerator at 6° to 8° C. for fifteen to eighteen hours, followed by water-bath at 38° C. for five minutes.	Antisheep amboceptor dilution (2 units).	Sheep corpuscles, 2 per cent.	Mix. Incubate in water-bath at 38° C. for one hour. Place tubes in refrigerator for a few hours and read results.
1.....	1 : 300	0.5 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.	
2.....	1 : 400	0.5 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.	
3.....	1 : 500	0.5 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.	
4.....	1 : 600	0.5 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.	
5.....	1 : 800	0.5 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.	
6.....	1 : 1000	0.5 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.	
7.....	1 : 1200	0.5 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.	
8.....	1 : 1600	0.5 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.	
9.....	1 : 2000	0.5 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.	
10.....	1 : 2400	0.5 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.	
11.....	0 .5 c.c. saline	0.5 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.	
12.....	1.0 c.c. saline	None	0.5 c.c.		0.5 c.c.	0.5 c.c.	

1. Prepare a 1 : 100 dilution as follows:

Equal parts hemolytic serum and glycerol	2 c.c.
Salt solution, 0.85 per cent	94 "
Phenol, 5 per cent	4 "

This remains active for several weeks if kept in the refrigerator.

2. For the titration make further dilutions in a series of 10 test-tubes and mix the contents of each tube thoroughly:

0.2 c.c. of 1 : 100 dilution + 1.8 c.c. saline = 1 : 1000 dilution
0.2 c.c. of 1 : 100 dilution + 3.8 c.c. saline = 1 : 2000 dilution
0.2 c.c. of 1 : 100 dilution + 5.8 c.c. saline = 1 : 3000 dilution
0.2 c.c. of 1 : 100 dilution + 7.8 c.c. saline = 1 : 4000 dilution
0.2 c.c. of 1 : 100 dilution + 9.8 c.c. saline = 1 : 5000 dilution
0.5 c.c. of 1 : 3000 dilution + 0.5 c.c. saline = 1 : 6000 dilution
0.5 c.c. of 1 : 4000 dilution + 0.5 c.c. saline = 1 : 8000 dilution
0.5 c.c. of 1 : 5000 dilution + 0.5 c.c. saline = 1 : 10,000 dilution
0.5 c.c. of 1 : 6000 dilution + 0.5 c.c. saline = 1 : 12,000 dilution
0.5 c.c. of 1 : 8000 dilution + 0.5 c.c. saline = 1 : 16,000 dilution

TITRATION OF HEMOLYSIN FOR KOLMER'S METHOD

Tube number.	Antisheep amboceptor 0.5 c.c.	Complement serum, diluted 1 : 30.	Sheep corpuscles, 2 per cent.	Salt solution, 0.85 per cent.
1.....	1 : 1000 dil.	0.3 c.c.	0.5 c.c.	1.7 c.c.
2.....	1 : 2000 dil.	0.3 c.c.	0.5 c.c.	1.7 c.c.
3.....	1 : 3000 dil.	0.3 c.c.	0.5 c.c.	1.7 c.c.
4.....	1 : 4000 dil.	0.3 c.c.	0.5 c.c.	1.7 c.c.
5.....	1 : 5000 dil.	0.3 c.c.	0.5 c.c.	1.7 c.c.
6.....	1 : 6000 dil.	0.3 c.c.	0.5 c.c.	1.7 c.c.
7.....	1 : 8000 dil.	0.3 c.c.	0.5 c.c.	1.7 c.c.
8.....	1 : 10,000 dil.	0.3 c.c.	0.5 c.c.	1.7 c.c.
9.....	1 : 12,000 dil.	0.3 c.c.	0.5 c.c.	1.7 c.c.
10.....	1 : 16,000 dil.	0.3 c.c.	0.5 c.c.	1.7 c.c.

Mix contents of tubes; incubate in a water-bath for one hour at 38° C., and read results. The highest dilution of hemolysin which in dose of 0.5 c.c. causes complete hemolysis is the amboceptor unit. Two units are employed in the various titrations and the test itself, and these are contained in 0.5 c.c. of a dilution twice as strong as that which gives the unit. Thus if the highest dilution which brings about complete hemolysis be 1 : 8000 (Tube 7) the amboceptor unit is 0.5 c.c. of a 1 : 8000 dilution, and the double unit used in the text is 0.5 c.c. of a 1 : 4000 dilution. When the proper dilution is found, enough should be prepared to conduct the complement titration and the test.

(3) **Titration of Complement for Kolmer's Method.**—In order to secure accurate adjustment of the hemolytic system this method requires that both amboceptor and complement be titrated each time tests are run. The latter titration is set up as soon as the former has been placed in the water-bath.

1. To 1 c.c. of the guinea-pig serum obtained as described on page 600 add 29 c.c. of salt solution, thus making a 1 : 30 dilution. Mix well.

2. Set up the complement titration as follows.

Tube No. 10 is the control of corpuscles and hemolysin and should show no hemolysis. Of the other tubes, some will show hemolysis, others

TITRATION OF COMPLEMENT FOR KOLMER'S METHOD

Tube number.	Complement serum dilution, 1 : 30.	Antigen (10 units).	Salt solution, 0.85 per cent.		Antisheep amboceptor (2 units).	Sheep corpuscles, 2 per cent.	
1.....	0.10 c.c.	0.5 c.c.	1.4 c.c.	Mix well. Place in water-bath at 38° C. for one hour.	0.5 c.c.	0.5 c.c.	Mix well. Incubate in water-bath at 38° C. for one hour. Read results.
2.....	0.15 c.c.	0.5 c.c.	1.4 c.c.		0.5 c.c.	0.5 c.c.	
3.....	0.20 c.c.	0.5 c.c.	1.3 c.c.		0.5 c.c.	0.5 c.c.	
4.....	0.25 c.c.	0.5 c.c.	1.3 c.c.		0.5 c.c.	0.5 c.c.	
5.....	0.30 c.c.	0.5 c.c.	1.2 c.c.		0.5 clc.	0.5 c.c.	
6.....	0.35 c.c.	0.5 c.c.	1.2 c.c.		0.5 c.c.	0.5 c.c.	
7.....	0.40 c.c.	0.5 c.c.	1.1 c.c.		0.5 c.c.	0.5 c.c.	
8.....	0.45 c.c.	0.5 c.c.	1.1 c.c.		0.5 c.c.	0.5 c.c.	
9.....	0.50 c.c.	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	
10.....	None	None	2.0 c.c.		0.5 c.c.	0.5 c.c.	

not. The tube with the least amount of 1 : 30 complement-serum which causes complete hemolysis is noted, and the tube containing the next higher amount is taken to indicate what Kolmer calls the "full unit" of complement. Twice this amount, or 2 full units, is taken as the dose to be used in the various titrations and in the test itself. After this dose has been determined the guinea-pig serum is further diluted so that the 2 full units are contained in 1 c.c. Kolmer advises the following scheme for finding the correct dilution: Divide 30 by the number of cubic centimeters which contain the dose of 1 : 30 dilution. The quotient is the dilution to be employed in a dose of 1 c.c. Thus, if the titration has shown first complete hemolysis in Tube 3, then the "full unit" is represented by Tube 4, containing 0.25 c.c. of the 1 : 30 dilution; and the dose, or 2 full units, is 0.5 c.c. of this dilution. Then 30 divided by 0.5 gives 60, and a 1 : 60 dilution should be made and 1 c.c. of this used as the dose in the test. The amount of this dilution to be prepared should be calculated from the number of tests to be run.

2. The Test Proper.—On the day before the test prepare the glassware; secure and wash the sheep corpuscles (p. 609); obtain the bloods to be tested (p. 610) as well as the guinea-pig blood to supply complement (p. 600). The test itself requires portions of two days, owing to the long incubation in the ice-box.

1. Prepare a 1 : 30 complement dilution and a 2 per cent. suspension of sheep corpuscles.

2. Titrate the hemolytic amboceptor and determine unit.

3. Titrate the complement and determine unit. This titration may be set up and placed in the water-bath as soon as incubation of the amboceptor titration has begun.

4. Inactivate the sera to be tested for fifteen minutes at 55° C.

5. In an appropriate rack arrange 6 tubes for each serum to be tested, including a known positive and a known negative serum; also 3 additional tubes for controls of antigen, hemolytic system, and corpuscles. Set up the test as indicated in the table on page 631, which shows the reagents to be placed in each tube. The following details may be helpful:

(*a*) The required dilutions of serum[1] are obtained as follows: Place 1.2 c.c. of physiologic salt solution in Tube No. 1 of each set; 0.5 c.c.

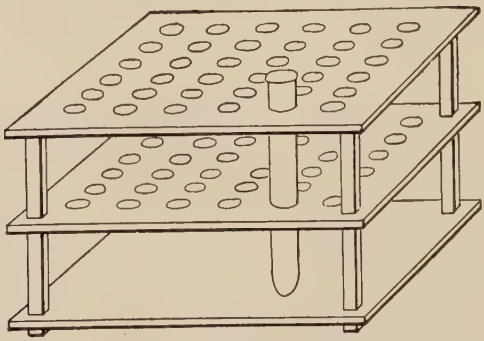


FIG. 294.—Convenient test-tube rack for Kolmer's method.

in Tubes No. 2, 3, and 5, and 2 c.c. in Tube No. 4. To Tube 1 add 0.3 c.c. of the serum to be tested, and mix well. From Tube 1 transfer 0.5 c.c. to Tube No. 2, and 0.5 c.c. to Tube 6. Mix; transfer 0.5 c.c. from Tube No. 2 to Tube No. 3. Mix Tube No. 3 and transfer 0.5 c.c. to Tube No. 4. Mix Tube No. 4, transfer 0.5 c.c. to No. 5, and discard 1.5 c.c. Mix Tube No. 5 and discard 0.5 c.c. Each of the serum tubes then contains a volume of 0.5 c.c. with the quantities of serum indicated in the table on page 631.

(*b*) The volume of antigen should in each case be 0.5 c.c., carrying 10 antigenic units, the appropriate dilution being determined by previous titration.

[1] When spinal fluid is tested the tubes should contain 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.5 c.c. (control) respectively; in each case being made up to a volume of 0.5 c.c. with saline.

"SET UP" FOR KOLMER'S QUANTITATIVE COMPLEMENT-FIXATION TEST FOR SYPHILIS

Tube number.	Patient's serum.	Antigen dilution (10 units).	Let stand five to thirty minutes.	Complement dilution (2 full units).	Mix. Incubate in refrigerator at 6° to 8° C. for fifteen to eighteen hours. Then warm in water-bath for five to ten minutes.	Antisheep amboceptor dilution (2 units).	Sheep corpuscles, 2 per cent.	Mix. Incubate in water-bath at 38° C. for one hour. Place in refrigerator one to three hours and read results.
1.....	0.1 c.c.	0.5 c.c.		1.0 c.c.		0.5 c.c.	0.5 c.c.	
2.....	0.05 c.c.	0.5 c.c.		1.0 c.c.		0.5 c.c.	0.5 c.c.	
3.....	0.025 c.c.	0.5 c.c.		1.0 c.c.		0.5 c.c.	0.5 c.c.	
4.....	0.005 c.c.	0.5 c.c.		1.0 c.c.		0.5 c.c.	0.5 c.c.	
5.....	0.0025 c.c.	0.5 c.c.		1.0 c.c.		0.5 c.c.	0.5 c.c.	
6 Serum control.....	0.1 c.c.	None.		1.0 c.c.		0.5 c.c.	0.5 c.c.	
7 Antigen control..	None.	0.5 c.c.		1.0 c.c.		0.5 c.c.	0.5 c.c.	
8 Hemolytic control..	None.	None.		1.0 c.c.		0.5 c.c.	0.5 c.c.	
9 Corpuscle control..	None.	None.		Saline only 2.5 c.c.		None.	0.5 c.c.	

Tubes 1 to 6 inclusive are set up for each of the sera to be tested, also for a known positive and a known negative serum. When only the *qualitative test* is desired, Tubes 2, 3, 4, and 5 are omitted. For spinal fluid the quantities in the first six tubes are 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.5 c.c. (control) of spinal fluid diluted in each case to a volume of 0.5 c.c.

(*c*) The tubes are allowed to stand for five to thirty minutes before adding complement.

(*d*) The volume of complement dilution in each of the first eight tubes is 1 c.c., carrying *two full units* of complement as determined by previous titration. In the ninth tube this is replaced by 2.5 c.c. saline.

6. Place the rack containing the test in a refrigerator at 6° to 8° C. for fifteen to eighteen hours. This temperature can usually be secured in the ice-compartment of an ordinary refrigerator. A thermometer should be placed in the rack with the tubes.

7. At the end of the ice-box incubation warm the tubes for five to fifteen minutes (never longer) in the water-bath, and add the antisheep amboceptor and the corpuscle suspension as indicated in the tables showing "set up" of the test.

8. Place the rack in the water-bath at 38° C. for one hour, then transfer to the refrigerator for one to three hours to allow the corpuscles to settle and make readings.

3. Reading and Recording Results.—All of the control tubes are first examined. The serum controls, the antigen control, and

the hemolytic control should show complete hemolysis. The corpuscle control should show none.

The degree of inhibition is recorded for the different quantities of each serum as follows:

0 = no inhibition
1 = + inhibition
2 = ++ inhibition
3 = +++ inhibition
4 = ++++ inhibition

The final record for each serum will then consist of five figures, one for each of the five different quantities of serum used. A typical record of a very strongly positive test might be 44420, indicating complete inhibition of hemolysis in the tube containing 0.1, 0.05, and 0.025 c.c. of serum, partial hemolysis in the tube containing 0.005 c.c. of serum, and complete hemolysis in the tube containing 0.0025 c.c. of serum.

Kolmer's experience with the method leads him to interpret the reactions as follows:

Partial or complete inhibition of hemolysis in the first four tubes: *Very strongly positive.*

Partial or complete inhibition of hemolysis in the first three tubes: *Strongly positive.*

Partial or complete inhibition of hemolysis in the first two tubes: *Moderately positive.*

Partial or complete inhibition of hemolysis only in the first tube: *Weakly positive.*

Complete hemolysis in all tubes: *Negative.*

F. Interpretation of Results

1. The Wassermann Reaction in Diseases Other than Syphilis.—For all practical purposes it may be accepted that fully positive reactions are limited to syphilis. There are, however, unquestioned records of occasional or even frequent positive reactions in the following diseases: yaws, relapsing fever, trypanosomiasis, pellagra, the tubercular form of leprosy, and scleroderma. Positive reactions, usually weak, have also occasionally been noted in persons suffering from diabetes with acidosis. These conditions are so

little likely to be mistaken for syphilis clinically, or are so rare in ordinary practice, that they are negligible as a source of error.

At one time scarlet fever was held responsible for many positive reactions, and there is still a wide-spread belief that tuberculosis very frequently causes them; but more recent studies have failed to confirm either belief. While weakly positive reactions doubtless sometimes occur in tuberculosis, Craig states that among many hundreds of tuberculous persons tested he has never found one *strongly positive* in which syphilis could be excluded with certainty; and Kolmer records the same experience with 250 cases of scarlet fever.

2. The Wassermann Reaction in Syphilis.—After infection takes place a certain time must elapse before an appreciable amount of the syphilitic substance upon which the reaction depends is produced. Therefore the reaction is usually negative or very weak in the early part of the primary stage. During the first two or three weeks after the appearance of the chancre the microscopic search for *Treponema pallidum* in tissue juice from the lesion has the greater diagnostic value. The serologic test should not, however, be entirely neglected in this early stage, since positive reactions have been obtained in exceptional cases even during the first week. The percentage of cases reacting positively rapidly increases until by the fifth week about 80 per cent. of the cases give a "three plus" or stronger reaction. In secondary syphilis a "four plus" reaction may be expected in practically every untreated case—certainly in more than 95 per cent. In treated cases the percentage is somewhat lower. Tertiary syphilis gives a somewhat smaller percentage of positives than does the secondary stage—about 80 to 85 per cent. of the general run of cases; but, since the protean and often obscure manifestations of the disease in this stage makes diagnosis by any other means very difficult, it is here that the complement-fixation test finds its greatest diagnostic value in general medicine.

In latent syphilis, with no clinical symptoms, the disease may have become so inactive that the syphilitic reagin is not formed in sufficient amounts to cause a reaction, or at most no more than a weakly positive one; nevertheless the published records show from 50 to 75 per cent. of latent cases—most of which have received considerable treatment—to react positively. The positive reaction

is many times the only recognizable sign of the disease at this stage. Of infants with symptoms of congenital syphilis, 90 to 100 per cent. give strongly positive reactions, while among those which do not develop symptoms until later, only about 40 to 45 per cent. give positives. Some infants with a positive reaction at birth never develop symptoms and the reaction soon becomes negative. In these cases it is assumed that there is transfer of the syphilitic reagin from the mother's blood to the child's without passage of the spirochetes themselves.

When syphilitic disease of the central nervous system is suspected both spinal fluid and blood are tested. Paresis almost invariably gives strong positives in both fluids. In locomotor ataxia and cerebral syphilis the reaction is somewhat less constant and distinctly weaker, being often positive only in the tubes containing the larger amounts of spinal fluid. In any case of syphilis, however early, a positive reaction with spinal fluid is strong evidence of involvement of the spinal cord or brain; and, at times, although not usually, this may be noted when the blood-serum is negative. With recent improvements in technic the delicacy and specificity of the test have increased, and it is probable that the complement-fixation test as it is now done in the better class of laboratories gives a still higher percentage of positives in all forms of syphilis.

3. Effect of Treatment.—As a rule, active treatment of any form of syphilis will reduce the strength of the reaction and may even cause it to become negative. Should the treatment be successful, the reaction will remain permanently negative; otherwise it will again become positive, usually within a few weeks after treatment is discontinued. The test is therefore generally relied upon to determine when a patient is fully cured, but for this purpose it must be repeated at intervals for several years after cessation of treatment. For such tests a sensitive antigen, such as cholesterolized extract, is especially desirable; and even a partial positive must be interpreted as indicating that living spirochetes are still present in the body.

Under certain circumstances treatment has an opposite effect: in patients in whom the reaction has been negative for a long time, as a result of earlier treatment or otherwise, a single dose of salvarsan will sometimes cause it to again become positive for a

short period. This is called the "provocative test," and by its aid it may sometimes be possible to detect latent syphilis in individuals who would otherwise be regarded as free. The positive reaction obtained in this way is evanescent and the time of its appearance varies, so that it is necessary to make the test daily for a week or ten days after administration of the salvarsan.

4. Summary of Interpretation.—(1) When the diseases mentioned on page 632 can be excluded a "four plus" or "strongly positive" reaction may be accepted with much confidence as indicating syphilis.

(2) "Three plus" reactions or the "moderately positive" of Kolmer usually mean syphilis, but must be interpreted in the light of circumstances. If obtained soon after the appearance of a suspicious primary lesion or during or soon after treatment they may be accepted as indicating syphilis.

(3) "Two plus" and "one plus" reactions are doubtful, and demand a repetition of the test. If either is obtained during or after treatment of a known syphilitic, it indicates continuance of the disease.

(4) A single negative result suggests the absence of syphilis, but does not prove it. The strength of the reaction often varies greatly from week to week; and it may even become temporarily negative spontaneously or, according to Craig, as a result of an alcoholic debauch. Two or three negatives at weekly or monthly intervals in persons who have never been under treatment for syphilis may, upon the other hand, be accepted with considerable confidence.

(5) Known cases of syphilis cannot be considered cured until the reaction has remained negative for several years after treatment has ceased.

COMPLEMENT-FIXATION TEST FOR GONORRHEA

Either of the methods given for syphilis may be used. The antigen is prepared from cultures of a great number of strains of gonococci and is best purchased from a biologic supply house. For use it is generally diluted 1 in 10 with saline, and is titrated each time tests are made. The dose used for the test is generally one-quarter to one-half the smallest amount that is anticomplementary, provided that this gives a strongly positive reaction with a known

positive serum, or with the antigonococcus serum marketed by biologic supply houses. The test is conducted with three different quantities of inactivated serum—0.05, 0.1, and 0.2 c.c.—together with corresponding controls in which no antigen is used. The primary incubation should be at least one hour in the water-bath or fifteen hours in the refrigerator.

The reaction is negative during the acute stage of gonorrhea, but is useful in determining the presence of a focus of chronic infection. In ordinary chronic gonorrheal urethritis the reaction is positive in about 35 per cent. of cases, while in gonorrheal arthritis the percentage is much higher, probably above 80 per cent. In general it may be said that the reaction is highly specific if properly carried out, but not very delicate, owing to the small amount of antibody present in the blood. A definitely positive reaction, therefore, is practically diagnostic; but the reactions are usually weaker than in syphilis, and a negative reaction does not exclude gonorrhea. A fact of much importance is that it becomes negative in a short time, usually two to four weeks, after a cure is effected.

COMPLEMENT-FIXATION TEST FOR TUBERCULOSIS

This may be performed in the same manner as the corresponding test for syphilis, with substitution of the tuberculosis antigen for the syphilitic. Either antihuman or antisheep methods may be employed. The antigen is best purchased ready prepared from a reliable biologic supply house.[1] The amount to be used in the test must be very carefully determined by titrations corresponding to those of the syphilis antigen, and preferably done on the day the main test is made. As a rule, one-third the anticomplementary unit should be used, provided this has good antigenic power. It is well to set up tubes with 0.05, 0.1, and 0.2 c.c. of the patient's serum, which should be inactivated. Primary incubation in the water-bath should be one to two hours, or, in the ice-box, twelve to eighteen hours.

As to the value of the complement-fixation test for tuberculosis

[1] For preparation of three widely used antigens see: Miller, H. R., and Zinsser, H.: Proc. Soc. Exper. Biol. and Med., vol. 13, p. 134, 1916. Petroff, S. A.: A Glycerin Extract of Tubercle Bacilli as an Antigen in Complement Fixation, Amer. Rev. Tuberc., vol. 2, p. 523, November, 1918. Fleisher, M. S., and Ives, G.: An Antigen for Complement Fixation in Tuberculosis, Jour. Lab. and Clin. Med., vol. 3, p. 302, February, 1918.

there is marked divergence of opinion. Some serologists give it much the same place as the corresponding test for syphilis, others believe that it has little value except as an interesting confirmatory test. Petroff, who is one of its strong advocates, has obtained 93.9 per cent. of positive reactions in clinically active tuberculosis; 56 per cent. in quiescent tuberculosis; 8.5 per cent. in apparently cured cases; 3.8 per cent. in normals; and 16.6 per cent. in other diseases supposedly without tuberculous complication. Many of the very far advanced cases react negatively.

III. FLOCCULATION TEST FOR SYPHILIS

In 1917 Meinicke, and later Sachs and Georgi, and Dreyer and Ward proposed tests for syphilis based upon the appearance of a white precipitate when an alcoholic extract of normal heart muscle (the "antigen" of the complement-fixation test for syphilis) is added to the blood-serum of a syphilitic individual. This is not a true precipitin reaction in the biologic sense, and in order to avoid confusion the name "flocculation test" is preferable to "precipitation test," which has been much used.

This test has been extended and modified in this country by Kahn, Herrold, and others. As carried out by Kahn the test, very briefly described, is as follows:

Reagents Required.—(*a*) *The Antigen.*—The antigen is most easily made from beef-heart powder prepared by the Digestive Ferments Company, Detroit. In a 250-c.c. Erlenmeyer flask place 25 c.c. of powdered beef-heart. Extract four times with ether by shaking for ten-minute periods with 100, 75, 75, and 75 c.c. of ether respectively. The ether is filtered off after each extraction, and the moist residue returned to the flask with a spatula. After the last extraction transfer the moist material to a sheet of white paper and dry until there is no odor of ether. Weigh the dry powder (usually 23 gm.), and add 5 c.c. of 95 per cent. alcohol for each gram. Shake the flask for ten minutes and continue extraction at room temperature for three days without shaking. At the end of this period shake the flask for five minutes and filter. Add 6 mg. of chemically pure cholesterol for each cubic centimeter of extract. Dissolve the cholesterol by rotating the flask in a water-bath at 37° C. Filter, and allow to stand one day before using. Corks covered with tinfoil should be used as stoppers for bottles.

Titration of Antigen.—The test is carried out with a mixture of cholesterolized antigen and salt solution. By titration is found the

minimum amount of physiologic salt solution which, added to the antigen, will produce a precipitate which will redissolve in salt solution. To this end mix antigen and salt solution in various proportions, as shown in the following table:

Tube number.	Antigen.	Salt solution.
1	1 c.c.	0.8 c.c.
2	1 "	0.9 "
3	1 "	1.0 "
4	1 "	1.1 "
5	1 "	1.2 "

A precipitate will form in each tube. Each antigen dilution is allowed to stand for thirty minutes, and is then tested for solubility as follows: 0.05, 0.025, and 0.0125 c.c. amounts, respectively, of each antigen dilution are pipeted with a 0.2-c.c. pipet graduated in 0.001 c.c. into three tubes (75 × 10 mm.). These small quantities must be placed in the bottom of the tubes. Add 0.15 c.c. of salt solution to each tube. Shake vigorously for three minutes, and add 0.5 c.c. salt solution to each tube, and observe for solution of the precipitate. Of the tubes whose precipitate readily dissolves in the salt solution, that containing the least amount of saline indicates the correct dilution of antigen to be used in the test. A good antigen is stable and does not need to be titrated again.

To dilute the antigen for use measure the required amounts of antigen and salt solution into two test-tubes, and mix by pouring the salt solution into the antigen, and then pouring back and forth a few times. The antigen must not be diluted longer than one-half hour before using in the test, and must stand ten minutes before using. Never dilute less than 1 c.c. of stock antigen; proportionately larger amounts may be used if necessary.

(*b*) *The Patient's Serum.*—This is rendered as clear as possible by centrifugation and is inactivated in a water-bath at 56° C. for thirty minutes.

(*c*) *Normal Saline.*—Prepare 0.85 per cent. sodium chlorid (C. P.) in distilled water.

The Test.—1. In three small test-tubes place 0.05, 0.025, 0.0125 c.c. of the freshly mixed antigen-saline. This necessitates use of 0.2 c.c. pipets graduated in thousandths of a cubic centimeter. The fluid should be deposited directly in the bottom of the tubes.

2. To each tube add 0.15 c.c. of the inactivated serum and mix.

3. Set up similar tubes with serum from a normal person and from a known syphilitic.

4. Shake the rack containing the tubes *vigorously* for three minutes.

5. Add 1 c.c. of salt solution to each tube containing 0.05 c.c. of antigen, and to the other tubes add 0.5 c.c. of salt solution. Make readings immediately. Stand before a window, hold the tubes as nearly horizontally as possible slightly above the level of the eyes, and look through the fluid against a dark background, such as a dark window shade.

6. Record results. A precipitate or clumps in a clear fluid indicates a *four-plus* reaction. If the fluid be not quite clear the reaction is recorded as *three-plus*. A precipitate in a cloudy fluid is *two-plus*. A weak precipitate in a cloudy fluid is *one-plus*. The record should show the readings of each of the three tubes. The final record of the test is the average of the three. If, for example, the three tubes were four-plus, two-plus, and negative, the test would be recorded as two-plus. A two-plus or higher reaction is considered positive.

The flocculation test has been studied in connection with the Wassermann reaction in a large number of cases. Undoubtedly it very closely parallels the Wassermann reaction, and the opinion of its advocates is that it is more sensitive. The chief difficulty is the recognition and interpretation of the slighter precipitates. The Kahn test has been adopted in the United States Navy as the standard serologic test for the diagnosis of syphilis.

CHAPTER X

BACTERIOLOGIC METHODS

Bacteriology has become so important a part of medicine that some knowledge of bacteriologic methods is imperative for the present-day practitioner. It has been the plan of this book to describe the various bacteria and bacteriologic methods with the subjects to which they seemed to be particularly related. The tubercle bacillus and its detection, for example, are described in the chapters upon Sputum and Urine; blood-cultures are discussed in the chapter upon Blood. There are, however, certain methods, notably the preparation of media and the study of bacteria by cultures, which do not come within the scope of any previous section, and an outline of these is given in the present chapter.

I. APPARATUS

Much of the apparatus of the clinical laboratory is called into use. Only the following need special mention:

1. **Sterilizers.**—Two are required.

The *dry*, or *hot-air sterilizer*, is a double-walled oven similar to the detached ovens used with gas and gasolene stoves. It has a hole in the top for a perforated cork with thermometer. The oven of any stove, even without a thermometer, will answer for many purposes. Ordinarily the heat should be sufficient to slightly brown but not char paper or cotton, and should be continued for one-half to one hour.

The *steam sterilizer* may be of the Arnold type, opening either at the top or the side. An *autoclave*, which sterilizes with steam under pressure, is very desirable, but not absolutely required. An aluminum pressure cooker (Fig. 295) is a very satisfactory substitute for the autoclave. It costs about twenty dollars.

2. **Incubator.**—This is the most expensive piece of apparatus which will be needed. It is made of copper, and has usually both a water- and an air-jacket surrounding the incubating chamber. It is provided with thermometer, thermoregulator, and some source

of heat, usually a Koch safety Bunsen burner if gas be used. With a little ingenuity one can rig up a drawer or a small box, in which a fairly constant temperature can be maintained by means of an electric light. The degree of heat can be regulated by moving the drawer in or out, or holes can be made in which corks may be inserted and removed as needed. A Thermos bottle has been suggested as a temporary makeshift. Upon occasion cultures may be kept warm by carrying them in an inside pocket.

The gas-heated copper incubators are now fast being displaced by the cheaper and more satisfactory wooden incubators in which electricity is the source of heat.

3. **Culture-tubes and Flasks.**—For most work ordinary test-tubes, 125 × 19 mm. without flange, are satisfactory. For special purposes a few 100 × 13 mm. and 150 × 19 mm. tubes may be needed. Heavy tubes, which do not easily break, can be obtained, and are especially desirable when tubes are cleaned by an untrained assistant. The tubes are usually stored in wire baskets.

Flasks of various sizes are needed. The Erlenmeyer type is best. Quart and pint milk bottles and 2-ounce, wide-mouthed bottles will answer for most purposes.

FIG. 295.—Aluminum pressure cooker, an efficient and comparatively inexpensive substitute for an autoclave.

4. **Platinum Wires.**—At least two of these are needed. Each consists of a piece of platinum wire about 8 cm. long, fixed in the end of a glass or metal rod. One is made of about 22-gage wire, and its end is curled into a loop 2 to 3 mm. in diameter. The other wire is somewhat heavier and its tip is hammered flat.

Lyon recommends the use of No. 20 nichochrome wire as nearly equal to platinum and very much cheaper. He makes a handle of No. 8, or thicker, aluminum wire, sawing an oblique notch in the end, inserting the nichochrome wire, and hammering the aluminum over it.

5. **Pipets.**—In addition to the graduated pipets with which every laboratory is supplied, there are a number of forms which are generally made from glass tubing as needed. One of the simplest of these is the "capillary pipet," made as follows: A section

of glass tubing, about 12 cm. long and 7 mm. in diameter, is grasped at the ends, and its center is heated in a concentrated flame. A blast-lamp is best, but a Bunsen burner will usually answer, particularly if fitted with a "wing" or "fish-tail" attachment. When the glass is thoroughly softened it is removed from the flame, and, with a steady but not rapid pull, is drawn out as shown in Figure 296. The slender portion is scratched near the middle with a file, and is broken to make two pipets, which are then fitted with rubber nipples. Two conditions are essential to success: the glass must be thoroughly softened, and it must be removed from the flame before beginning to pull.

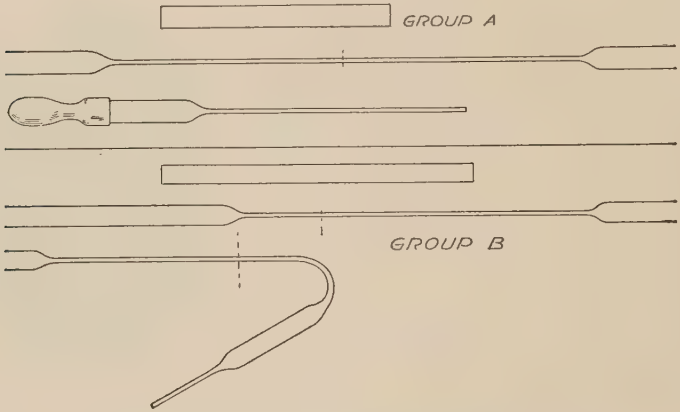


Fig. 296.—Process of making capillary pipets (Group A) and Wright's capsule (Group B). The dotted lines indicate where the glass is to be broken.

A nipple can be made of a short piece of rubber tubing, one end of which is plugged with a glass bead.

This pipet has many uses about the laboratory. With a grease-pencil mark about 2 cm. from its tip (Fig. 302), it is useful for measuring very small quantities of fluid, as in making dilutions for the Widal test and in counting bacteria in vaccines. Mett's tubes for pepsin estimation may be made from the capillary portion. The capillary portion also makes a very satisfactory blood-lancet if the center is heated in a low flame and the two ends pulled quickly apart.

Another useful device is the Wright capsule, which is made as shown in Figure 296. Its use is illustrated in Figure 283. After the straight end is sealed, the curved portion may be hooked over

the aluminum tube of the centrifuge, and the contained blood or other fluid sedimented; but the speed should not be so great as to break the capsule.

II. STERILIZATION

All apparatus and materials used in bacteriologic work must be sterilized before use.

Glassware and metal are heated in the hot-air sterilizer at 150° C. for one hour, at 180° C. for half an hour, or at 200° C. for five minutes. Flasks, bottles, and tubes are plugged with cotton before heating. Petri dishes may be wrapped in paper in sets of three. Pipets and all-glass hypodermic syringes are placed in cotton-stoppered test-tubes.

Culture-media and other fluids must be sterilized by steam. Exposure in an autoclave to a temperature of 110° C. (6 pounds' pressure) for one-half hour or of 121° C. (about 15 pounds' pressure) for fifteen minutes is generally sufficient. Sometimes 20 pounds for twenty minutes will be required to kill resistant spores. In using the autoclave it is extremely important that all the air be allowed to escape, so that the sterilizing chamber may be filled with superheated steam. With the Arnold sterilizer the intermittent plan must be adopted, since steam at ordinary pressure will not kill spores. This consists in steaming for thirty to forty-five minutes on three or four successive days. Spores which are not destroyed upon the first day develop into the vegetative form and are destroyed at the next heating. Gelatin media must not be exposed to steam for more than twenty minutes at a time, and must then be removed from the sterilizer and cooled in cold water, otherwise the gelatin may lose its power to solidify. Media containing sugars are generally sterilized in the Arnold, since great heat tends to break down the sugar.

Cotton and **gauze** are sterilized by either hot-air or steam, preferably the latter.

III. PREPARATION OF CULTURE-TUBES

New tubes should be washed in a very dilute solution of nitric acid, rinsed in clear water, and allowed to drain dry.

Tubes which contain dried culture-media are cleaned with a test-tube brush after boiling in a 1 or 2 per cent. solution of washing-soda. They are then rinsed successively in clear water, acidulated water, and clear water, and allowed to drain.

The well-known **bichromate cleaning fluid** is very valuable for cleaning glassware of all kinds. It consists of:

Potassium bichromate, technical	100 gm.
Concentrated sulphuric acid, technical	250 c.c.
Water	750 "

Glassware may be placed in this solution for one day or longer and then rinsed thoroughly and dried.

The tubes are now ready to be plugged with raw cotton—the "cotton batting" of the dry-goods stores. This is done by pushing a wad of cotton into each tube to a depth of about 3 cm. with a glass rod. The plugs should fit snugly, but not too tightly, and should project from the tube sufficiently to be readily grasped by the fingers. The tubes are next placed in wire baskets and heated in an oven for about one-half hour at 150° C. in order to mold the stoppers to the shape of the tubes. The heating should not char the cotton, although a slight browning does no harm. The tubes are now ready to be filled with culture-media.

IV. CULTURE-MEDIA

For a careful study of bacteria a great variety of culture-media is required, but only a few—bouillon, agar, and solidified blood-serum—are much used in routine work. A great deal of work can be done with a single medium, for which purpose Huntoon's "hormone agar" is probably best. The ordinary culture-media, put up in tubes ready for use, can be purchased through any pharmacy. A long list of media, including practically all formulæ in general use, and many others, is now offered in powder form under the name Bacto Dehydrated Media. These have only to be dissolved in a specified amount of water and sterilized. Most of them, at least, are very satisfactory, and their use greatly facilitates the work of a small laboratory.

Preparation of Culture-media.—

Beef Infusion

Hamburger steak, lean	500 gm.
Tap-water	1000 c.c.

Mix well; let soak about twenty-four hours in an ice-chest, and squeeze through cheese-cloth. This infusion is not used by itself, but forms the basis for various media. "Double strength" infusion, used in making agar-agar, requires equal parts of the meat and water.

Infusion Bouillon

Beef infusion	1000 c.c.
Peptone (Witte)	10 gm.
Salt	5 "

Boil until dissolved; bring to original volume with water; adjust reaction, and filter.

Beef Extract Bouillon

Liebig's extract of beef	3 gm.
Peptone	10 "
Salt	5 "
Tap-water	1000 c.c.

Heat until all ingredients are dissolved, cool, and beat in the whites of two eggs; bring slowly to the boiling-point again, boil briskly for five minutes, and filter. It is not usually necessary to adjust the reaction.

Dextrose Brain Broth

This is an excellent fluid-medium for growing streptococci. It is used to advantage in making blood-cultures, the indicator at times showing growth before colonies appear on plates. Dissolve 8 gm. dehydrated "Bacto" nutrient broth (Digestive Ferments Company, Detroit), and 8 gm. of salt in 1 liter boiling water. Cool, add 2 gm. C. P. dextrose, and 1 c.c. Andrade indicator (p. 650). The medium is tubed in tall tubes (8 x 5/8 inches). Three small pieces of calf's brain and two or three pieces of marble are added to each tube, and the medium is then sterilized in the autoclave at 20 pounds' pressure for twenty minutes.

Beef Infusion Agar

Preparation of this medium usually gives the student much trouble. There should be no difficulty if the directions are carefully carried out.

Agar-agar, powdered or in shreds	15 gm.
Tap-water	500 c.c.

Boil until thoroughly dissolved and add—

Peptone	10 gm.
Salt	5 "

When these have dissolved, replace the water lost in boiling, cool to about 60° C., and add 500 c.c. double-strength beef infusion. Bring slowly to the boil, adjusting the reaction meanwhile, and boil for at least five minutes. Filter while *hot* through a moderately thick layer of absorbent cotton wet with *hot* water in a *hot* funnel. A piece of coarse wire gauze should be placed in the funnel underneath the cotton to give a larger filtering surface. This medium will be clear enough for ordinary work. If an especially clear agar is desired, it can be filtered through paper in an Arnold sterilizer.

Beef Extract Agar

This is made by boiling 15 gm. of powdered agar in 1000 c.c. of beef extract bouillon until dissolved, replacing the water lost in boiling, and filtering through paper in a sterilizer. It can be cleared with egg if desired. This makes a 1.5 per cent. agar. For some purposes, as when beef extract agar is used as a base for Endo's medium, twice the amount of the powdered agar is required.

Glycerol Agar

To 1000 c.c. melted agar add 60 to 70 c.c. glycerol, and mix well.

N. N. N. Medium

Mix 14 gm. agar, 6 gm. salt, and 900 c.c. water. Prepare as plain agar medium, tube and sterilize in the autoclave. For use melt the agar and when cooled to 48° C. add one-third volume of defibrinated blood.

Petroff's Medium

This medium is now very generally used for growing tubercle bacilli. Mix 500 gm. chopped lean beef, or veal, with 500 c.c. of 15 per cent. glycerol solution. Place in the ice-box for twenty-four hours; filter through gauze, and to each 100 c.c. of filtrate add 1 c.c. of 1 per cent. alcoholic solution of gentian violet. Sterilize the shells of six eggs by placing them in 70 per cent. alcohol for ten minutes. Carefully crack the eggs, and mix the whites and yolks in a sterile beaker. Add an equal amount (about 200 c.c.) of the glycerol beef extract, and mix well. Tube, placing 4 c.c. in each tube. Inspissate at 85° C. until medium is solidified, and on the two succeeding days heat for one hour to 75° C.

Sabouraud's Agar for Fungi

This is a widely used standard medium for cultivation of yeasts and molds, particularly the fungi of the parasitic skin diseases. Ashford recommends it for isolation of *Monilia psilosis* from the feces. By virtue of its acid reaction it retards the growth of bacteria.

Maltose or glucose	4.0 gm.
Peptone	1.0 "
Agar	1.5 "
Water	100.0 c.c.

Adjust reaction by titration to +2.

Tartaric Acid Medium for Fungi

Another medium recommended for fungi because it inhibits the growth of ordinary bacteria is made as follows:

Tartaric acid	25 gm.
Dextrose	50 "
Water, to make	100 c.c.

Sterilize in the autoclave twenty minutes at 20 pounds' pressure. Add 10 c.c. of this sterile tartaric-acid-dextrose solution to 190 c.c. of melted agar, and pour plates

Blood Agar

Blood agar is now widely used for culturing sputum and other material in which the pneumococcus or streptococcus may be expected.

Sterile agar in a flask or a series of tubes is melted, cooled to 45° to 50° C., and maintained at this temperature in a water-bath. Sterile human or rabbit blood which may be citrated or defibrinated is then added in the proportion of 1 c.c. of blood to 4 or 5 c.c. of agar, and well mixed by rotating. For the study of hemolysis by the streptococcus the proportion of blood should not exceed 1 in 10. The medium is then poured into Petri plates, or, if in tubes, is cooled in a slanted position, and is incubated to make sure of its sterility. When extreme economy is essential the blood-agar may be poured upon the surface of plain agar slants.

Human blood may be obtained from an arm vein by means of the device shown in Figure 99, which has been charged with 1 or 2 c.c. of 10 per cent. sodium citrate before sterilization. If defibrinated blood be preferred, a number of small pieces of broken

glass tubing are substituted for the citrate, and the tube is gently shaken. Rabbits' blood is secured from the heart.

Serum, Ascitic, and Hydrocele Agar

These are especially useful for the pneumococcus, streptococcus, and gonococcus.

To tubes containing 4 or 5 c.c. of nutrient agar, which has been melted, cooled to 45° to 50° C., and kept at this temperature in a water-bath, add 1 or 2 c.c. of sterile blood-serum, hydrocele fluid, or ascitic fluid. Mix well by rotating and cool in a slanted position.

When these albuminous fluids cannot be secured in a sterile condition, they may, as has been suggested by Grace, be rendered slightly alkaline with 10 per cent. sodium hydroxid solution, and sterilized in the autoclave. They will not coagulate and may be added to agar as required. This is then titrated and brought to the correct reaction with hydrochloric acid, and finally sterilized. We have had excellent results with such media.

Hormone Agar (Huntoon)

This has now become a very popular stock-medium for general bacteriologic procedures. The modification proposed by Sadie Bailey is given: Thoroughly wash 15 gm. of agar-agar shreds in running water, and dissolve by boiling in 1 liter of distilled water. Cool to 50° or 60° C. Add 500 gm. ground lean beef or beef-heart, heat to boiling, and cook slowly for from fifteen to twenty minutes. Filter through an ordinary round flour-sieve, about 16 mesh. Add peptone, 10 gm., and sodium chlorid 5 gm., and boil for five minutes. Correct the reaction to pH 7.5. Allow the precipitate to settle out and decant the clear supernatant medium. Add from 0.25 to 1 per cent. dextrose as desired, tube, and sterilize either by heating for one hour in the Arnold sterilizer on three successive days, or in the autoclave for twenty minutes at 15 pounds' pressure. When citrated blood is added to this medium it is especially useful in culturing pneumococci and streptococci. Gonococci will also grow on hormone blood-agar.

Endo's Agar

Prepare a 3 per cent. beef-extract agar (p. 646). Neutralize to phenolphthalein, sterilize, and store in 100-c.c. quantities.

To make Endo's medium melt 100 c.c. of this agar, add 1 gm. chemically pure lactose, and 0.5 c.c. of fuchsin-sulphite solution prepared as follows: To 10 c.c. of a 10 per cent. solution of anhydrous sodium sulphite add 2 c.c. of a 10 per cent. alcoholic solution of basic fuchsin, and heat for a few minutes.

The finished medium may be sterilized in the Arnold, but too great heat is to be avoided because of the danger of breaking up the sugar. The medium is red when hot, faintly pink or colorless when cold.

Lactose fermenters (colon bacilli, and so forth) give red colonies on Endo's medium, although the color may not appear in twenty-four hours' incubation; others (typhoid, paratyphoid, and dysentery bacilli) give colorless or gray colonies.

Teague's medium is preferred by some workers. It is quite similar to Endo's agar except that 0.5 per cent. saccharose and 0.5 per cent. lactose are added in place of 1 per cent. lactose, and in place of the fuchsin-sulphite solution to each 100 c.c. of medium are added 2 c.c. of 2 per cent. aqueous solution of yellowish eosin and 2 c.c. of 0.5 per cent. solution of methylene-blue.

Lactose Litmus Agar

Melt 100 c.c. of nutrient agar such as is used for Endo's medium, add enough azolitmin solution to give a distinct lilac color, and 5 c.c. of hot freshly prepared 20 per cent. solution of pure lactose. Tube and sterilize, preferably in the Arnold.

Russell's Double Sugar Agar

Make a 2 to 3 per cent. beef extract agar, adjust reaction by titration to about +0.7, and add azolitmin solution to a purple violet color. Now neutralize to azolitmin, and to each 100 c.c. add 1 gm. pure lactose and 0.1 gm. pure dextrose. The sugars are dissolved in a little water before adding. Mix well, tube, sterilize in the Arnold, and cool in a slanted position. There should be a deep butt below the slant.

This medium is used to differentiate organisms of the colon-typhoid group. It is customary to inoculate tubes both with a surface streak and with a stab made from the same colony. Colon bacilli turn both slant and butt red with gas-bubbles in the butt. Typhoid and dysentery bacilli give a grayish growth on the violet

slant and a deep pink in the butt without bubbles. The growth of paratyphoid bacilli, A and B, resembles that of typhoid except that there is gas production in the butt.

Instead of azolitmin most workers prefer using 1 per cent. of Andrade's indicator. The final reaction of the agar should be about pH 7.2. If the reaction is satisfactory, the medium will have a red color when hot and will be practically colorless when cold. Andrade's indicator is made by adding a 4 per cent. solution of sodium hydroxid to a 0.5 per cent. solution of acid fuchsin until the color changes from red to orange or yellow. As the color change takes place slowly, some time must elapse between additions. The usual proportion is about 16 c.c. of the alkali to 100 c.c. of the dye solution.

Fig. 297.—Dunham's fermentation tube, consisting of a small tube inverted in a standard culture-tube. When a liquid culture-medium is present the air in the inner tube is driven off during sterilization.

Gelatin

Dissolve 100 to 120 gm. "golden seal" gelatin in 1000 c.c. nutrient bouillon with as little heat as possible, adjust the reaction, cool, beat in the whites of two eggs, bring slowly to the boiling-point, boil for a few minutes, and filter hot through filter-paper wet with hot water. Sterilize in an Arnold sterilizer for twenty minutes upon three successive days and cool in cold water after each heating. Keep at room temperature between heatings.

Sugar Media

Any desired sugar may be added to bouillon, agar, or gelatin in proportion of 10 gm. to the liter. It is best added in solution. Dextrose is most frequently required. When other sugars are added, media made from beef-extract should be used, since those made from beef-infusion contain enough dextrose to cause confusion.

The various sugars may also be added to Dunham's peptone medium and Hiss' serum-water-litmus.

For the study of gas production the sugar media are placed in U tubes or, better, in Dunham's fermentation tubes (Fig. 297). These consist of standard culture-tubes with small tubes, about 8 by 25 mm., inverted inside of them. The culture-medium is introduced

to a depth of about $1\frac{1}{2}$ inches. The air in the inner tube is driven off during sterilization. As a rule, sugar media are sterilized in the Arnold, as sugar does not resist heat well.

Löffler's Blood-serum

Dextrose-bouillon (1 per cent.)	1 part
Blood-serum	3 parts

Mix and tube. Place in an inspissator at the proper slant for three to six hours at 80° to 90° C. When firmly coagulated, sterilize in the usual way. A large "double-cooker" makes a satisfactory inspissator. The tubes are placed in the inner compartment upon a layer of cotton at the proper slant, a lid with perforation for a thermometer is applied, and the whole is weighted down in the water of the outer compartment. An Arnold sterilizer with the door left ajar also makes a good inspissator.

Blood-serum is obtained as follows: Beef or pig blood is collected in a bucket at the slaughter-house and placed in an ice-chest until coagulated. The clot is then gently loosened from the wall of the vessel. After about twenty-four hours the serum will have separated nicely and can be siphoned off. It is then stored in bottles with a little chloroform until needed. Red cells, if abundant, darken the medium, but do no harm.

This has long been the approved medium for the diphtheria bacillus. For this purpose Greenspon has improved it by including 1 c.c. of 50 per cent. sodium citrate solution in each 100 c.c. of mixture, and then adjusting the reaction with 3 per cent. citric acid to pH 6.4, using bromthymol-blue as an indicator. The advantages are that Gram-positive cocci are inhibited and diphtheria bacilli grow more luxuriantly.

Egg Medium

This has been recommended as a substitute for solidified blood-serum. In a mortar grind thoroughly the white and yolk of one egg with 10 to 15 c.c. of 1 per cent. dextrose bouillon. Place in tubes, inspissate, and sterilize as described for solidified blood-serum.

Cystine Medium for B. Tularense (Francis)

The stock medium is made of beef infusion to which is added 1 per cent. peptone, 0.5 per cent. salt, and from 1 to 1.5 per cent.

agar-agar. The adjusted reaction should be pH 7.6. To this stock medium is added 0.1 per cent. cystine and 1 per cent. glucose. It is necessary to heat the medium for fifteen minutes in streaming steam in an Arnold sterilizer to melt the agar and to sterilize the cystine. After cooling to 45° C. add 5 per cent. sterile horse-serum. The medium is tubed and incubated for twenty-four hours to insure sterility.

Litmus Milk

Fresh milk is steamed in an Arnold sterilizer for half an hour, and placed in the ice-chest overnight. The milk is siphoned off from beneath the cream, and sufficient aqueous solution of litmus or, preferably, azolitmin is added to give a bluish-violet color. It is then tubed and sterilized.

Potato

Cylinders about $\frac{1}{2}$ inch in diameter are cut from potato and split obliquely. These wedge-shaped pieces are soaked overnight in running water and placed, broad ends down, in large tubes, in the bottom of which is placed a little cotton saturated with water. They are sterilized for somewhat longer periods than ordinary media.

Dunham's Peptone Solution

Peptone	10 gm.
Salt	5 "
Water	1000 c.c.

Dissolve by boiling; filter, tube, and sterilize.

This medium is used to determine indol production. To a twenty-four- to forty-eight-hour-old culture is added 5 to 10 drops of concentrated, chemically pure sulphuric acid and 1 c.c. of 1 : 10,000 solution of sodium nitrite. Appearance of a pink color shows the presence of indol. A pink color before the nitrite is added shows the presence of both indol and nitrites.

Hiss' Serum-water Media

Blood-serum	1 part
Water, about	3 parts

Steam in the Arnold for fifteen minutes to destroy any diastase that may be present. Adjust reaction to +0.2 to +0.8. Add litmus or azolitmin solution to give a bluish-violet color. Finally,

add 1 per cent. of inulin or any desired sugar and sterilize in the Arnold. Should the medium solidify, too large a proportion of serum was used. Inulin should be added in solution, and, since it usually contains spores, should first be sterilized in the autoclave. The inulin medium is very useful in distinguishing between the pneumococcus and streptococcus.

Bile Medium

Ox- or pig-bile is obtained at the slaughter-house, tubed, and sterilized. This is used especially for growing typhoid bacilli from the blood during life and, filtered as clear as possible, for the solubility test which distinguishes between pneumococci and streptococci. The following is probably as satisfactory as fresh bile and is more convenient:

Dehydrated ox-bile ("Bacto")	30.0 gm.
Peptone	2.5 "
Water	250.0 c.c.

Dissolve, place in tubes, and sterilize.

Reaction of Media.—The chemical reaction of the medium exerts a marked influence upon the growth of bacteria. It is adjusted after all ingredients are dissolved by adding sufficient caustic soda solution to overcome the acidity of the meat and other substances used. In general, the most favorable reaction lies between the neutral points of litmus and phenolphthalein, representing a very faint alkalinity to litmus. The reaction may be adjusted by titration, or by the newer colorimetric method.

Titration Method.—After all ingredients are dissolved and the loss during boiling has been replaced with water, 10 c.c. of the medium are transferred to an evaporating dish, diluted with 40 c.c. of water, and boiled for three minutes to drive off carbon dioxid. One c.c. of 0.5 per cent. alcoholic solution of phenolphthalein is then added, and decinormal sodium hydroxid solution is run in from a buret until the neutral point is reached, indicated by the appearance of a permanent pink color. The number of cubic centimeters of decinormal solution required to bring this color indicates the number of cubic centimeters of *normal* sodium hydroxid solution which will be required to neutralize 100 c.c. of the medium. The standard reaction of the American Public Health Association for bacteriologic examination of water and milk is +1,

which means that the medium must be of such degree of acidity that 1 c.c. of normal solution would be required to neutralize 100 c.c. This corresponds to faint alkalinity to litmus. Most pathogenic bacteria grow better with a reaction of +0.5 to +1. Example: If the 10 c.c. which were titrated required 2 c.c. of decinormal solution to bring the pink color, the reaction is +2, and 1 c.c. of normal sodium hydroxid must be added to each 100 c.c. of the medium to reduce it to the standard, +1.

Colorimetric Method.—With the growing recognition of the importance of exact adjustment of the true reaction or hydrogen-ion concentration of media for the optimum growth of many bacteria, titration has been largely discarded as failing to give a sufficiently accurate index, owing partly to the many buffer substances which are present, and partly to the difficulty in getting an exact and uniform end-point. Accurate determination of hydrogen-ion concentration by means of the potentiometer is out of the question for clinical laboratories owing to the great cost of the apparatus. The colorimetric method is therefore now generally relied upon, and is entirely sufficient for practical purposes. This depends upon the fact that the indicator, bromthymol blue, gives a series of color tints at different hydrogen-ion concentrations within the range required for this work, that is, from pH 6 to pH 7.6. With this indicator the color is yellow at pH 6; at pH 6.2 the color is yellowish-green up to pH 7; at pH 7 the color becomes bluish-green, and deepens to bright blue at pH 7.6.

Reagents Required.—(*a*) Bromthymol blue, 0.04 per cent. alcoholic solution.

(*b*) Normal sodium hydroxid and decinormal sodium hydroxid, the latter made from the former by accurate dilution. These are best kept in flasks which have been lightly coated inside with paraffin.

(*c*) A series of standard phosphate solutions of varying hydrogen-ion concentrations prepared according to Sorensen as follows:

One-fifteenth molecular acid or primary potassium phosphate. Dissolve 9.078 gm. of the pure crystalline salt, KH_2PO_4, in freshly distilled water and make up to 1000 c.c.

One-fifteenth molecular alkaline or secondary sodium phosphate. Expose the pure recrystallized salt, $Na_2HPO_412(H_2O)$ to the air for from ten days to two weeks, protected from dust. Ten molecules of water are given off and a salt of the formula $Na_2HPO_42(H_2O)$ is obtained. Dissolve 11.876 gm. of this in freshly distilled water and make up to 1000 c.c.

To make standard solutions of different hydrogen-ion concentrations in a graduated series mix the two phosphate solutions in the proportions shown in the following table:

pH	6.4	6.6	6.8	7.0	7.1	7.2	7.3	7.4	7.5	7.6	7.7	7.8	8.0	8.2	8.4
Primary potassium phosphate, c.c.	73	63	51	37	32	27	23	19	15.8	13.2	11	8.8	5.6	3.2	2.0
Secondary sodium phosphate, c.c.	27	37	49	63	68	73	77	81	84.2	86.8	89	91.2	94.4	96.8	98.0

Place 10-c.c. quantities of these standard solutions in a series of Pyrex or Non-Sol glass test-tubes, which must be of equal diameter as shown by the 10 c.c. of fluid reaching to the same height in each. Add 0.5 c.c. of the 0.04 per cent. bromthymol blue indicator to each tube, and seal with a paraffined cork. Sets of standard color tubes covering any pH range can be purchased ready prepared together with empty tubes of the same diameter.

Method.—1. Place the standard color-tube representing the pH value desired in one of the end holes of the front row of the comparator shown in Figure 298. If the desired value falls between two of the standards, place these in the end holes of the front row. Place a tube of the untreated medium back of each.

2. In a test-tube of the same diameter place 10 c.c. of the medium to be adjusted, agar or other solid medium being liquefied by heat. Add 0.5 c.c. of the 0.04 per cent. bromthymol blue solution, and mix.

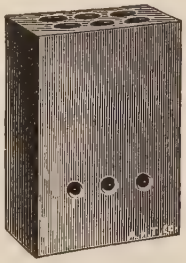


FIG. 298.—A color comparator (Clark's model) for use in adjusting reaction of culture-media by the colorimetric method.

3. From an accurate buret add decinormal sodium hydroxid solution a very little at a time, mixing after each addition, until the color matches that of the standard chosen, or falls between the standards next above or below. For comparison the tube is placed in the middle hole of the front row of the comparator with a tube of water back of it. Note the amount of decinormal solution required to bring the 10 c.c. of medium to the desired reaction. This amount is then the amount of *normal* sodium hydroxid solution which must be added to 100 c.c. of the medium to secure this reaction. After this has been added, the reaction should again be determined as a check. Most media, particularly those containing sugars, become slightly more acid during sterilization; and it is well when great accuracy is desired to make a check determination upon a 10-c.c. portion of the finished and sterilized product which has been kept out for the purpose.

The optimum hydrogen-ion concentration for growth of the more important bacteria, as determined by Fennel and Fisher, is as follows:

Pneumococcus	7.8
Streptococcus	7.6–7.8
Meningococcus	7.6
Gonococcus	7.5–7.6
Bacillus typhosis and B. paratyphosis	6.2–7.2
Bacillus dysenteriæ	6.3–7.8
Bacillus influenzæ	7.8–8.0

Tubing Culture-media.—The finished product is stored in flasks or distributed into test-tubes. This is done by means of a funnel fitted with a section of rubber tubing with a glass tip and a pinch-cock. Great care must be exercised, particularly with media which solidify, not to smear any of them upon the inside of the mouth of the tube, otherwise the cotton stopper will stick. Tubes are generally filled to a depth of 3 or 4 cm. For stab-cultures a greater depth is required.

After tubing all culture-media must be sterilized, as already described. Agar-tubes are cooled in a slanting position to secure the proper surface for inoculation.

Storage of Culture-media.—All media should be stored in a cool place, preferably an ice-chest. Evaporation may be prevented by covering the tops of the tubes with tin-foil or with the rubber caps which are sold for the purpose; or the cotton stopper may be pushed in a short distance and a cork inserted.

V. STAINING METHODS

In general, bacteria are stained to determine their morphology, their reaction with special methods (for example, Gram's method), and the presence or absence of certain structures, as spores, flagella, and capsules. Staining methods for various purposes have been given in previous chapters and can be found by consulting the Index. The formulæ of the staining fluids are given in the Appendix.

Method of Staining for Morphology.—The following method is used when one wishes to detect the presence of bacteria or to study their morphology. It is applicable both to films from cultures and to smears from pus or other pathologic material. Any simple bacterial stain may be used, but Löffler's methylene-blue or Pappenheim's pyronin-methyl-green will generally be found more satisfactory.

1. Make a thin smear upon a slide or cover-glass. Heavy wax-pencil marks across the slide will limit the stain to any portion desired.

2. Dry in the air, or by warming high above the flame, where one can comfortably hold the hand.

3. "Fix" by passing the preparation, film-side up, rather slowly through the flame of a Bunsen burner: a cover-glass three times, a slide about twelve times. One can learn to judge the proper temperature by touching the glass to the back of the hand at intervals. If the film takes on a brownish discoloration, most marked about the edges, it has been scorched and is worthless. Smears can also be fixed by flaming with alcohol, as described for blood-films (p. 271), or by soaking for one to three minutes in a 1 per cent. solution of mercuric chlorid and rinsing well. The last avoids all possibility of spoiling the preparation by scorching.

4. Apply the stain for the necessary length of time, generally one-quarter to one minute.

5. Wash in water.

6. Dry by waving high above a flame or by blotting with filter-paper.

7. Mount by pressing the cover, film-side down, upon a drop of Canada balsam or immersion-oil on a slide. Slides may be examined with the oil-immersion lens without a cover-glass.

Gram's Method.—This is a very useful aid in differentiating certain bacteria and should be frequently resorted to. It is very easy and should not be the bugbear which it apparently is to many students. It depends upon the fact that when treated successively with gentian-violet and iodin certain bacteria (owing to formation of insoluble compounds) retain the stain when subsequently treated with alcohol, whereas others quickly lose it. The former are called *Gram-positive;* the latter, *Gram-negative.* In order to render Gram-negative organisms visible some contrasting counterstain is commonly applied, but this is not a part of Gram's method proper. The following modification of Gram's method, suggested by G. H. Ruhland, has been in daily use in the Mayo Clinic for the last sixteen years:

1. Make smears, dry, and fix by heat or mercuric chlorid (p. 270).

2. Cover the preparation for half a minute with a 2 per cent. solution of crystal violet in methyl alcohol of the highest purity. This stain is much more satisfactory than the unstable anilin gentian-violet originally used.

3. Wash with water.

4. Apply Gram's iodin solution one-half minute.

5. Wash in alcohol until the purple color ceases to come off. This is conveniently done in a watch-glass. The preparation is placed in the alcohol, face downward, and one edge is raised and lowered with a needle. As long as any color is coming off, purple streaks will be seen diffusing into the alcohol where the surface of the fluid meets the smear. If forceps be used, beware of stain which may have dried upon them. The thinner portions of smears from pus should be practically colorless at this stage. Microscopically, the nuclei of pus-corpuscles should retain little or no color. *If, with proper technic, Gram-positive organisms are decolorized, the fault probably lies in the iodin solution, which tends to become acid when long exposed to light. It can be corrected by adding a pinch of sodium bicarbonate to the bottle of solution.* Within recent years acetone, suggested by Lyon, has come into extensive use, in place of alcohol, as a decolorizer. It acts very quickly and is generally very satisfactory.

6. Apply a contrast stain for one-half to one minute. The stains formerly used for this purpose were an aqueous or alcoholic solution of Bismarck brown or a weak solution of fuchsin. A 1 per cent. aqueous solution of safranin is very much better. In the writers' experience, Pappenheim's pyronin-methyl-green mixture if properly made is also satisfactory; it brings out Gram-negative bacteria sharply, and is especially desirable for intracellular Gram-negative organisms like the gonococcus and influenza bacillus, since the bacteria are bright red and nuclei of cells blue.

7. Wash in water, dry, and mount.

The more important bacteria react to this staining method as follows:

Gram Staining (Deep purple).	Gram Decolorizing (Colorless unless a counterstain be used).
Staphylococcus.	Gonococcus.
Streptococcus.	Meningococcus.
Pneumococcus.	Micrococcus catarrhalis.
Bacillus diphtheriæ.	Bacillus of influenza.
Bacillus tuberculosis.	Typhoid-dysentery-colon group.
Bacillus of anthrax.	Spirillum of Asiatic cholera.
Bacillus of tetanus.	Bacillus pyocyaneus.
Bacillus aërogenes capsulatus.	Bacillus of Friedländer.
	Koch-Weeks bacillus.
	Bacillus of Morax-Axenfeld.

Möller's Method for Spores.—Bodies of bacteria are blue, spores are red.

1. Make thin smears, dry, and fix.
2. Wash in chloroform for two minutes.
3. Wash in water.
4. Apply 5 per cent. solution of chromic acid one-half to two minutes.
5. Wash in water.
6. Apply carbolfuchsin and heat to boiling.
7. Decolorize in 5 per cent. solution of sulphuric acid.
8. Wash in water.
9. Apply 1 per cent. aqueous solution of methylene-blue one-half minute.
10. Wash in water, dry, and mount.

Huntoon's Method for Spores.—This method is simple and appears to be very reliable. Spores are deep red, bodies of bacteria are blue.

1. Make a rather thick smear, dry, and fix in the usual way.
2. Apply as much of the stain as will remain on the cover-glass, and steam over a flame for one minute, replacing the stain lost by evaporation.
3. Wash in water. The film is bright red.
4. Dip the preparation a few times into a weak solution of sodium carbonate (7 or 8 drops of saturated solution in a glass of water). Too long application of the carbonate will cause the spores to be blue.
5. The instant the film turns blue, rinse well in water.
6. Dry, mount, and examine.

Preparation of Stain.—

1. Acid fuchsin (Grübler) ..... 4 gm.
   Aqueous solution acetic acid (2 per cent.) ..... 50 c.c.

2. Methylene-blue (Grübler) ..... 2 gm.
   Aqueous solution acetic acid (2 per cent.) ..... 50 c.c.

Mix the two solutions, let stand for fifteen minutes, and filter off the voluminous precipitate through moistened filter-paper. The filtrate is the staining fluid. It keeps several weeks, but requires filtration when a precipitate forms.

Löffler's Method for Flagella.—The methods for flagella are applicable only to cultures. Enough of the growth from an agar-

culture (which should not be more than eighteen to twenty-four hours old) to produce faint cloudiness is added to distilled water. A small drop of this is placed on a cover-glass, spread by tilting, and dried quickly. The covers must be absolutely free from grease. To insure this they may be warmed in concentrated sulphuric acid, washed in water, and kept in a mixture of alcohol and strong ammonia. When used they are dried upon a fat-free cloth. Covers may be dried without touching them with the fingers by rubbing between two blocks of wood covered with several layers of lint-free cloth.

1. Fix by heating the cover over a flame while holding in the fingers.
2. Cover with freshly filtered mordant and gently warm for about a minute.

The mordant consists of:

Aqueous solution of tannic acid (20 per cent.)	10 c.c.
Saturated solution ferrous sulphate, cold	5 "
Saturated aqueous or alcoholic solution gentian-violet	1 "

3. Wash in water.
4. Apply freshly filtered anilin-gentian-violet, warming gently for one-half to one minute.
5. Wash in water, dry, and mount in balsam.

Caspule staining methods are given on pages 73 and 74.

VI. METHODS OF STUDYING BACTERIA

The purpose of bacteriologic examinations is to determine whether bacteria are present or not, and, if present, their species and comparative numbers. In general, this is accomplished by: (1) direct microscopic examination; (2) cultural methods; (3) animal inoculation.

1. Direct Microscopic Examination.—Every bacteriologic examination should begin with a microscopic study of smears from the pathologic material, stained with a general stain, by Gram's method, and often by the method for the tubercle bacillus. This yields a great deal of information to the experienced worker, and in many cases is all that is necessary for the purpose in view. It will at least give a general idea of what is to be expected, and may determine future procedure.

2. Cultural Methods.—(1) **Collection of Material.**—Material for examination must be collected under absolutely aseptic conditions. It may be obtained with a platinum wire—which has been heated to redness just previously and allowed to cool—or with a swab of sterile cotton on a stiff wire or wooden applicator. Such swabs may be placed in cotton-stoppered test-tubes, sterilized, and kept on hand ready for use. Fluids which contain very few bacteria, and hence require that a considerable quantity be used, may be collected in a sterile hypodermic syringe or one of the pipets described on page 642. The method for blood-cultures is given on page 305; for sputum, page 64; for urine, page 201; for feces, page 435.

(2) **Inoculating Media.**—The material is thoroughly distributed over the surface of some solid medium, Huntoon's hormone agar being probably the best for routine work. Blood-agar is preferable for streptococci and pneumococci. Special media are required for the gonococcus, tubercle bacillus, and the hemophilic group. When previous examination of smears has shown that many bacteria are to be expected, a second tube should be inoculated from the first, and a third from the second, so as to obtain isolated colonies in at least one of the tubes. The platinum wire must be heated to redness *before* and *after* each inoculation. When only a few organisms of a single species are expected a considerable quantity of the material is mixed with a fluid medium.

(3) **Incubation.**—Cultures are placed in an incubator which maintains a uniform temperature, usually of 37.5° C., for eighteen to twenty-four hours, and the growth, if any, is studied as described later. Gelatin will melt with this degree of heat, and must be incubated at about room temperature.

(4) **Study of Cultures.**—When the original culture contains more than one species, they must be separated, or obtained in "pure culture," before they can be studied satisfactorily. This must be done as soon as possible, since some pathogenic organisms quickly die out in cultures. To accomplish this it is necessary to so distribute them on solid media that they form separate colonies, and to inoculate fresh tubes from the individual colonies. In routine work the organisms can be sufficiently distributed by drawing the contaminated wire over the surface of the medium in a series of streaks. If a sufficient number of streaks be made, some

of them are sure to show isolated colonies. Another method of obtaining isolated colonies is to inoculate the water of condensation of a series of tubes, the first from the second, the second from the third, etc., and, by tilting, to flow the water once over the surface of the medium. One or more of these tubes will be almost sure to show nicely separated colonies.

In order to determine the species to which an organism belongs it is necessary to consider some or all of the following points:

(*a*) Naked-eye and microscopic appearance of the colonies on various media.

(*b*) Comparative luxuriance of growth upon various media. The influenza bacillus, for example, can be grown upon media containing hemoglobin, but not on the ordinary media.

(*c*) Morphology, special staining reactions, and the presence or absence of spores, flagella, and capsules. Staining methods for these purposes have been given.

(*d*) Motility. This is determined by observing the living organism with an oil-immersion lens in a hanging-drop preparation, made as follows: A small drop of a bouillon culture or of water of condensation from an agar or blood-serum tube is placed upon the center of a cover-glass; and over this is pressed the concavity of a "hollow-ground slide" previously ringed with vaselin. The slide is then turned over so as to bring the cover-glass on top. In focusing, the edge of the drop should be brought into the field. Great care must be exercised not to break the cover by pushing the objective against it.

It is not always easy to determine whether an organism is or is not motile, since the motion of currents and the Brownian motion which affects all particles in suspension are sometimes very deceptive.

(*e*) Production of chemical changes in the media. Among these are coagulation of milk; production of acid in milk and various sugar media to which litmus has been added to detect the change; production of gas in sugar media, the bacteria being grown in fermentation tubes similar to those used for sugar tests in urine; and production of indol.

(*f*) Ability to grow without free oxygen.

(*g*) Effects produced when inoculated into animals.

(5) **Anaërobic Methods.**—Some bacteria, the "obligate anaërobes," will not grow unless free oxygen is excluded. This may be accomplished in various ways. Perhaps the most convenient is the following method of J. H. Wright: After the culture-medium in the test-tube has been inoculated, push the cotton stopper in until its top is about 1.5 cm. below the mouth of the tube. Fill in the space above the stopper with dry pyrogallic acid, and pour on it just enough strong solution of sodium hydroxid to dissolve it. Finally, insert a rubber cork and seal with paraffin.

3. Animal Inoculation.—In clinical work this is resorted to chiefly to detect the tubercle bacillus. The method is described on page 541.

For the study of bacteria in cultures a small amount of a pure culture is injected subcutaneously or into the peritoneal cavity. The animals most used are guinea-pigs, rabbits, and mice. For intravenous injection the rabbit is used because of the easily accessible marginal vein of the ear.

VII. CHARACTERISTICS OF VARIOUS IMPORTANT BACTERIA

Owing to the great number of bacterial species, most of which have not been adequately studied, positive identification of an unknown organism is often a very difficult problem. Fortunately, however, only a few are commonly encountered in routine work, and identification of these with comparative certainty presents no great difficulty. Their more distinctive characteristics are outlined in this section.

1. Staphylococcus pyogenes aureus.—The morphology and staining reactions (p. 523) and the appearance of the colonies are sufficient for diagnosis. Colonies on solidified blood-serum and agar are rounded, slightly elevated, smooth and shining, and vary in color from light yellow to deep orange. Young colonies are sometimes white.

2. Staphylococcus pyogenes albus.—This is similar to the above, but colonies are white. It is generally less virulent.

3. Staphylococcus pyogenes citreus.—The colonies are lemon yellow; otherwise it resembles the white staphylococcus.

4. Streptococcus.—The morphology and staining reactions have been described (p. 523). The chains are best seen in the water of condensation and in bouillon cultures. The cocci are not

motile. Colonies on blood-serum are minute, round, grayish, and translucent. Litmus milk is usually acidified and coagulated, although slowly.

When cocci of the morphology of streptococci are found it is first necessary to distinguish them from pneumococci to which they are closely related. Their colonies on ordinary media are similar. The following points will probably be sufficient:

Capsule Formation.—The great majority of streptococci do not possess capsules. Pneumococci usually show them in pus or in other material fresh from the animal body, or when grown on a serum-medium.

Fermentation of Inulin.—The pneumococcus generally coagulates and acidifies Hiss' serum-water-litmus-inulin within twenty-four hours. The streptococcus rarely ferments inulin and then only after four or five days. The writer believes this to be an extremely valuable differential point.

Solubility in Bile.—Pneumococci are soluble in bile, streptococci are not. The test is carried out as follows: To a twenty-four-hour broth culture of the organism add one-tenth to one-fifth its volume of sterile 10 per cent. solution of sodium taurocholate in physiologic salt solution or of ox-bile medium (p. 653) which has been filtered as clear as possible and sterilized. Place in the incubator for one hour. If the organism is bile soluble the turbidity of the culture will clear up, and subcultures made from this culture will not show growth.

The streptococci have been much studied of late, and a great number of strains have been separated upon the basis of carbohydrate fermentation. From the clinical and pathologic point of view, it is doubtful whether such a classification offers any advantage over the simpler one based upon the appearance of the growth upon blood agar. This distinguishes three groups:

Hemolytic Streptococci.—The colonies on blood agar, after twenty-four or forty-eight hours, are surrounded by a clear colorless zone 2 to 4 mm. wide, due to hemolysis. This group includes most of the virulent strains, such as *Streptococcus pyogenes* and *Streptococcus anginosus*. In liquid media they tend to grow in long chains.

Green-producing Streptococci.—The colonies on blood agar are surrounded after twenty-four or forty-eight hours by a cloudy,

greenish zone about a millimeter wide. These are commonly included under the name *Streptococcus viridans*. They are less actively virulent than the hemolyzing type and often associated with mild, chronic inflammations. Certain non-pathogenic strains are also included. In liquid media they tend to grow in short chains.

Anhemolytic Streptococci.—These cause no change in the surrounding medium. The group is practically limited to non-pathogenic strains.

It is important to remember that these reactions on blood agar depend largely upon the use of a proper medium. The optimum reaction of the agar is about pH 7.4, and not more than one-tenth its volume of blood should be added. The plates should be freshly poured in order that the surface may be moist.

5. Pneumococcus.—The only organism with which this is likely to be confused is the streptococcus. The distinction, which is sometimes difficult, is described above.

The morphology and staining of the pneumococcus have been described (p. 72). In cultures it frequently forms long chains. Capsules are not present in cultures except upon special media. They show best upon a serum-medium like that described for the gonococcus, but can frequently be seen in milk. Colonies on blood-serum resemble those of the streptococcus. Colonies on blood agar show a green zone like those of *Streptococcus viridans*. The pneumococcus usually promptly acidifies and coagulates milk and acidifies and coagulates Hiss' serum-water with inulin. The very valuable "bile solubility" test is described in the section upon the Streptococcus.

Upon the basis of immunologic reactions four types of pneumococci are now recognized. Types I and II are the typical pneumococci. Type III has large distinct capsules and viscid colonies, and is known as *Pneumococcus mucosus*. It has some of the characteristics of the streptococcus and was formerly known as *Streptococcus mucosus capsulatus*. Strains have been found which hemolyzed blood, did not ferment inulin, and were not bile soluble. Type IV includes miscellaneous strains which cannot be placed in the other groups. The pneumococci of the normal mouth are most frequently of this type. For Types I, II, and III specific immune sera can be prepared by injecting suspensions of killed pneumococci into appropriate animals (horses, rabbits). These sera are ex-

tremely useful in the laboratory for determining the type of newly isolated organisms, and Type I serum has great value in the treatment of pneumonia.

In 454 cases of lobar pneumonia Avery, Chickering, Cole, and Dochez found the incidence of the four types to be as follows: Type I, 33.3 per cent.; Type II, including three subtypes, 33.5 per cent.; Type III, 13 per cent.; Type IV, 20.3 per cent.

Determination of Pneumococcus Types.—When pure cultures of the organism are at hand, the type is readily determined by means of agglutination tests with immune sera of Types I, II, and III. Encapsulated Gram-positive cocci which are bile soluble and are not agglutinated by any of the three are assigned to Type IV. Clinically, typing is generally undertaken in order that the patient, if his organisms prove to be of Type I, may have early benefit of the curative serum. Time is lacking for careful culturing and isolation of the organism by the usual methods. Several methods are available, two of which are given below. In the first of these inoculation of mice is resorted to. Pneumococci are very pathogenic for mice and outgrow practically all other organisms, and appear in the blood in pure culture. The other method, which is not available in all cases, extracts the antigen from the sputum, and this is at once identified by precipitin tests without need of isolation of the pneumococcus.

Method of Avery, Chickering, Cole, and Dochez.—A small mass of the sputum, as fresh as possible, is washed gently in several changes of sterile saline. The mass is then ground in a sterile mortar with 1 c.c. of sterile saline and injected into the peritoneal cavity of a white or gray mouse. The mouse will appear sick within five to twenty-four hours. A small drop of peritoneal exudate is then obtained by peritoneal puncture with a sterile capillary pipet through a slight incision in the skin, and smears are prepared. Should these show only a moderate growth of pneumococci, or if other organisms are present in considerable numbers, time should be allowed for further growth. If there is an abundant growth of pneumococci, the mouse is killed, tied out upon a board, and the body opened aseptically. Cultures upon blood-agar are made from the heart's blood and peritoneal exudate, and also smears from the latter to be stained by Gram's method and a capsule stain. The peritoneal cavity is then thoroughly washed out with 4 or 5 c.c. of sterile saline by means of a capillary pipet and the washings collected

in a centrifuge tube. The agglutination test and precipitin tests are then applied to these washings as described in the following paragraphs. The cultures from the heart's blood and the peritoneal exudate are carried through in the usual way, the organism isolated, and confirmatory agglutination tests applied.

Agglutination Test.—This may be applied to the peritoneal washings mentioned above or to any pure culture of the pneumococcus in broth. The fluid is centrifugalized for a few minutes at low speed to throw down leukocytes and débris, and the supernatant fluid which contains the bacteria is pipeted into another centrifuge tube. This is centrifugalized for a long time at very high speed to throw down the pneumococci. The supernatant fluid, which should be as clear as possible, is pipeted into another tube to be used for the precipitin test, and the sediment, which consists largely of bacteria, is mixed with just enough saline to give a turbid suspension. Agglutination[1] and bile-solubility tests are then set up in a series of five small test-tubes (about 8 x 50 mm.), as follows:

Tube 1—Bacterial suspension, 0.5 c.c. + Type I serum, diluted 1 : 20, 0.5 c.c.
Tube 2—Bacterial suspension, 0.5 c.c. + Type II serum, undiluted, 0.5 c.c.
Tube 3—Bacterial suspension, 0.5 c.c. + Type II serum, diluted 1 : 20, 0.5 c.c.
Tube 4—Bacterial suspension, 0.5 c.c. + Type III, serum, diluted 1 : 5, 0.5 c.c.
Tube 5—Bacterial suspension, 0.5 c.c. + Sterile ox-bile or 10 per cent. sodium taurocholate, 0.1 c.c.

The tubes are placed in the incubator for one hour. Agglutination is read macroscopically as has been described for the Widal test. Bile solubility is recognized by the clearing up of the turbidity in Tube 5. The tube in which agglutination occurs indicates the type of the pneumococcus under examination, since its type must correspond to that of the serum which agglutinates it. Strains which are agglutinated by Type II serum undiluted (Tube 2), but not by the same serum diluted 1 : 20 (Tube 3), belong to one of the subgroups (a, b, x) of Type II. Strains which are soluble in bile, but do not agglutinate in any tube, are placed in Type IV.

Precipitin Test.—This is applicable to the peritoneal washings mentioned above, and is undertaken when there are so many contaminating organisms as to make the agglutination test uncertain. It may fail if too much saline was used for the washings. The fluid must be cleared as much as possible by long centrifugation at high speed. The test is set up as follows:

[1] Immune sera for these tests may be obtained from the biologic supply houses.

Tube 1—Peritoneal washings, 0.5 c.c. + Type I serum, diluted 1 : 10, 0.5 c.c.
Tube 2—Peritoneal washings, 0.5 c.c. + Type II serum, undiluted, 0.5 c.c.
Tube 3—Peritoneal washings, 0.5 c.c. + Type II serum, diluted 1 : 10, 0.5 c.c.
Tube 4—Peritoneal washings, 0.5 c.c. + Type III serum, diluted 1 : 5, 0.5 c.c.

A positive reaction in any tube is shown by the appearance of a white cloud. The interpretation is the same as that given above for the agglutination test.

Rapid Method of Oliver.—This method is applicable only when satisfactory samples of sputum, containing many pneumococci, can be obtained, which, however, is possible in nearly all typical cases of lobar pneumonia. The type of pneumococcus present can be determined within one-half to three-quarters of an hour. Briefly the method consists in treating the sputum with bile to dissolve the pneumococci, and thus liberate its specific precipitable substances, and then in applying the precipitin test with the several specific antisera.

1. Select the portion of sputum most likely to contain many pneumococci. This is usually the most purulent or blood-streaked portion. Make a smear and stain by Gram's method to confirm the presence of the pneumococcus.

2. Place 1 to 2 c.c. of this sputum in a conical centrifuge tube, and add 3 to 5 drops of 10 per cent. sodium taurocholate or of undiluted sterile ox-bile.

3. Thoroughly break up and mix the mass by stirring with a rod or grinding in a small mortar, meanwhile adding, a few drops at a time, just enough sterile physiologic saline to insure sufficient fluidity to allow of centrifugation. This should require less than 1 c.c. of saline.

4. Place the tube in a water-bath at 42° to 45° C. for twenty minutes.

5. Centrifugalize at high speed until the supernatant fluid is as clear as possible.

6. Carefully pipet 0.3 to 0.5 c.c. of the supernatant fluid to each of 3 small absolutely clean test-tubes (about 8 x 50 mm.). Label tubes 1, 2, and 3.

7. To tubes 1, 2, and 3 add 1 to 2 drops of Types I, II, and III pneumococcus antiserum, respectively. A positive test is evidenced by an almost immediate clouding and flocculation, which is increased by heating in a water-bath at 42° C. for fifteen minutes. The tube in which this occurs indicates the type of the pneumococcus present in the sputum. When no reaction occurs in any tube, the organism is assumed to be of Type IV, provided it is, in reality, a pneumococcus.

8. When only a very small quantity of sputum is available it is desirable to centrifugalize in correspondingly narrow tubes. The three precipitin tests may be carried out with as little as 0.3 c.c. of supernatant fluid.

6. Micrococcus catarrhalis grows readily at room temperature and on ordinary media, where it forms large, white, dry colonies with irregular edges and elevated centers. This readily distinguishes it from the gonococcus and meningococcus, which it closely resembles in morphology and staining reactions.

7. Gonococcus.—Its morphology and staining peculiarities are given on page 524. These usually suffice for its identification, cultural methods being rarely undertaken. In cultures the chief diagnostic point is its failure to grow on ordinary media. To grow it a satisfactory medium is ascitic or hydrocele agar, or blood agar made from human or rabbit blood. Colonies are minute, grayish, and translucent.

8. Diplococcus intracellularis meningitidis.—It grows poorly or not at all on plain agar. On Löffler's blood-serum, upon which it grows fairly well, colonies are round, colorless or hazy, flat, shining, and viscid looking. Upon serum, ascitic, hydrocele, and blood agar meningococci form faint bluish colonies 1 to 2 mm. in diameter, distinctly larger than the usual streptococcus colony. It quickly dies out. Its morphology and staining reactions are given on page 538.

9. Diphtheria Bacillus.—The diagnosis is usually made from a study of stained smears from eighteen-hour cultures upon Löffler's blood-serum, grown for twelve to eighteen hours. Its morphology and staining peculiarities are then characteristic (p. 544). The bacilli are non-motile and Gram-positive. The colonies are round, elevated, smooth, and grayish.

10. Typhoid, Dysentery, Colon Group.—This is a large group, the more important members of which are the typhoid bacillus, the paratyphoid bacilli, A and B, the several types of dysentery bacilli, and the colon bacilli. They are medium-sized, Gram-negative, non-spore-bearing rods. They grow well on ordinary media and form rounded, grayish, slightly elevated, viscid-looking colonies. Those mentioned here do not liquefy gelatin, although gelatin is readily liquefied by the proteus group which is closely related.

Most of the members of the group may be easily and definitely identified by agglutination with specific immune sera, if such be available. However, owing to the occurrence of group reactions, it is necessary to perform the test with various dilutions of serum.

Next to the agglutination tests the fermentation reactions may be relied upon. These are indicated in the table below. For diagnostic work certain special media are in general use, such as Endo's medium, which is used especially for plating directly from

CHARACTERISTICS OF THE PRINCIPAL MEMBERS OF TYPHOID, DYSENTERY, COLON GROUP

	Fermentation of carbohydrates.							
	Dextrose.	Lactose.	Saccharose.	Maltose.	Mannite.	Xylose.	Indol formation.	Motility.
Bacillus typhosus.....	a	0	0	a	a	x	—	+
B. paratyphosus A....	ag	0	0	ag	ag	0	—	+
B. paratyphosus B....	ag	0	0	ag	ag	a	—	+
B. dysenteriæ, Shiga...	a	0	0	0	0	x	—	—
B. dysenteriæ, Flexner.	a	0	0	a	a	x	+	—
B. dysenteriæ, Hiss-Russell, "Y".....	a	0	0	0	a	x	+	—
B. coli communis.....	ag	ag	0	ag	ag	x	+	+
B. coli communior....	ag	ag	ag	ag	ag	x	+	+
B. acidi lactici.....	ag	ag	0	ag	ag	x	+	—
B. lactis aërogenes....	ag	ag	ag	ag	ag	x	+	—
B. paracoli.....	ag	0	ag	ag	ag	x	?	+

Explanation: a, acid produced. +, present or positive.
ag, acid and gas produced. —, absent or negative.
0, no reaction. x, not required for identification.

the feces or urine, and Russell's double sugar agar, which is used for rapid presumptive differentiation between typhoid, paratyphoid, and colon bacilli, after they have been obtained in pure culture. For the fermentation tests given in the table the carbohydrate is

dissolved in sugar-free broth, 1 gram to 100 c.c., 1 per cent. of Andrade's indicator (or enough azolitmin solution to give a bluish color) is added, and the medium is tubed in fermentation tubes.

11. Bacillus of Influenza.—Diagnosis will usually rest upon the morphology and staining peculiarities, described on page 74, and upon the fact that the bacillus will not grow on ordinary media, but does grow upon hemoglobin-containing media. It can be grown upon agar-slants which have been smeared with a drop of blood from a puncture in the finger. Before inoculation these slants should be incubated to make sure of sterility. The colonies are difficult to see without a hand lens. They are very minute, discrete, and transparent, resembling small drops of dew.

12. Bacillus of Tuberculosis.—The methods of identifying this important organism have been given (pp. 64, 200). Cultivation is not resorted to in routine clinical work. It grows very slowly and only on certain media. It is Gram-positive and non-motile.

13. Bacterium tularense.—This organism is the cause of a new American disease, tularemia, described by Francis.[1] The disease occurs in nature as a fatal bacteremia of various rodents, especially rabbits. It is transmitted to man either by handling diseased carcasses or through the bite of a blood-sucking insect or tick. Characteristic symptoms are glandular enlargement, often with suppuration, and typhoid-like fever. The diagnosis is best made by a blood-serum agglutination test using *B. tularense* antigen, which may be obtained from the Hygienic Laboratory, Washington, D. C. The organism is a small, non-motile Gram-negative bacillus, from 0.3 to 0.7 μ long; it also appears as a coccus. It does not grow on ordinary media, but will grow readily on special media such as that described on page 651.

VIII. BACTERIOLOGIC CLASSIFICATION

There has been much confusion in bacteriologic nomenclature since Nägeli, in 1857, first used the class name Schizomycetes. Various systems of classification have been proposed, until recently the Committee on Characterization and Classification of the Society of American Bacteriologists has proposed a new classification, which has not been adopted universally, but which is taught in

[1] Francis, Edward: Tularemia, Francis, 1921. A New Disease of Man, Washington, Government Printing Office, 1922, Hygienic Laboratory, Bulletin 130, 84 pp.

SYNONYMS PROPOSED FOR NAMES OF BACTERIA

Old name.	New name proposed by Society of American Bacteriologists (Bergey, 2d edition).	Mentioned on page
Actinomyces hominis	Actinomyces hominis	62
Bacillus, acidi-lactici	Escherichia acidi-lactici	670
" Boas-Oppler	Lactobacillus boas-oppleri	407
" bulgaricus	Lactobacillus bulgaricus	407
" coli	Escherichia coli	670
" coli Communis	Escherichia coli	670
" coli Communior	Escherichia Communior	670
" diphtheria	Corynebacterium diphtheriæ	669
" dysentery	Eberthella paradysenteriæ	670
" Friedländer	Klebsiella pneumoniæ	74
" fusiform	Fusiformis dentium	460
" influenzæ	Hemophilus influenzæ	74
" Kochs-Weeks	Hemophilus conjunctivitidis	550
" lactis aërogenes	Aërobacter aërogenes	670
" of leprosy	Mycobacterium lepræ	547
" paratyphosus A	Salmonella paratyphi	670
" pertussis	Hemophilus pertussis	75
" proteus vulgaris	Proteus vulgaris	200
" pyocyaneus	Pseudomonas æruginosa	200
" smegma	Mycobacterium smegmatis	69
" tubercle	Mycobacterium tuberculosis	64
" typhoid	Eberthella typhi	306
" xerosis	Corynebacterium xerosis	550
Bacterium tularense	Pasteurella tularensis	671

SYNONYMS PROPOSED FOR NAMES OF BACTERIA—*Continued*

Old name.	New name proposed by Society of American Bacteriologists (Bergey, 2d edition).	Mentioned on page
Diplococcus intracellularis meningitidis	Neisseria intracellularis	668
Diplococcus, Morax-Axenfeld	Hemophilus lacunatus	550
Gonococcus	Neisseria gonorrhœæ	200
Leptospira icterohemorrhagiæ	Leptospira icterohemorrhagiæ	461
" icteroides	Leptospira icteroides	461
Leptothrix buccalis	Leptotrichia buccalis	56
Micrococcus catarrhalis	Neisseria catarrhalis	75
" Ureæ	Micrococcus ureæ	199
Nocardia (streptothrix)	Actinomyces farcinicus	76
Pneumococcus	Diplococcus pneumoniæ	665
" mucosus	Diplococcus pneumoniæ	665
Spirochæta bronchialis	Borrelia bronchialis	460
" dentium	Treponema microdentium	462
" recurrentis	Borrelia recurrentis	459
" refringens	Borrelia refringens	462
" vincenti	Borrelia vincenti	460
Staphylococcus pyogenes albus	Staphylococcus albus	663
" pyogenes aureus	Staphylococcus aureus	663
" pyogenes citreus	Staphylococcus citreus	663
Streptococcus, hemolytic	Streptococcus pyogenes	664
" viridans	Streptococcus mitor	664
" anhemolytic	Streptococcus anhemolyticus	665
Treponema pallidum	Treponema pallidum	557
" pertenue	Treponema pertenuis	462

many schools. The student is referred for details especially to the work of Enlows, Bergey and Buchanan.[1] The short table of synonyms (pages 672 and 673) gives the familiar term used in this text on the page indicated and the name proposed for the organism in the new classification.

[1] Enlows, M. A.: The Generic Names of Bacteria, Washington, Government Printing Office, 1920, Hygienic Laboratory, Bulletin 121, September. Bergey, D. H.: Manual of Determinative Bacteriology, Baltimore, Williams and Wilkins, 1925, 462 pp. Buchanan, R. E.: General Systematic Bacteriology, Baltimore, Williams and Wilkins, 1925, 597 pp.

CHAPTER XI

VACCINES. BIOLOGICAL SKIN TESTS

Bacterial vaccines, sometimes called "bacterins," which within recent years have come to play an important rôle in therapeutics, are suspensions of definite numbers of dead bacteria in normal salt solution. While in many cases, notably in gonorrhea and tuberculosis, ready prepared or "stock" vaccines are satisfactory, it is usually desirable and often imperative for best results to use vaccines which are especially prepared for each patient from bacteria which have been freshly isolated from his own lesion. These latter are called "autogenous vaccines." Only through them can one be certain of getting the exact strain of bacterium which is producing the disease.

I. PREPARATION OF VACCINE

1. Preparation of Materials.—A number of $\frac{1}{2}$-ounce bottles with medium wide mouths are thoroughly cleaned and sterilized by baking. Each is charged with 9.8 c.c. of freshly filtered physiologic salt solution, and is capped with paper which is folded neatly about the neck and held in place with a rubber band. The bottles and contents are sterilized in an autoclave at 20 pounds' pressure for fifteen minutes, together with an equal number of rubber "vaccine-bottle caps" and a pair of forceps wrapped in paper or gauze. If an autoclave be not available, three daily steamings in an Arnold sterilizer will answer. The rubber caps are then applied with great care to avoid contamination. The bottles are kept on hand as "blanks" (Fig. 299), which are later converted into vaccines by addition of killed bacteria and a preservative.

When desired, 2-ounce bottles may be used with 50 c.c. of salt solution. In such cases ordinary rubber nursing nipples without holes make excellent caps. They are applied as indicated in Figure 300.

A number of test-tubes, each charged with 10 c.c. of physiologic salt solution and plugged with cotton, are also prepared and sterilized.

2. Obtaining the Bacteria.—A culture on an appropriate solid medium is made from the patient's lesion, and a pure culture is obtained in the usual way. This preliminary work should be carried through as quickly as possible in order that the bacteria may not lose virulence by long growth upon artificial media. If for any reason there is much delay, it is best to begin over again, the experience gained in the first trial enabling one to carry the second through more rapidly. When a pure culture is obtained, a number of tubes of blood-serum or agar—10 or 12 in the case of streptococcus or pneumococcus, 4 or 5 in the case of most other organisms—are planted and incubated overnight or until a good growth is obtained.

3. Making the Suspension.—A few cubic centimeters

FIG. 299.—Vaccine bottle with rubber cap. The bottle holds 15 c.c.

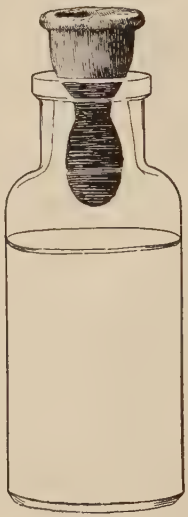


FIG. 300.—Showing the use of a nursing nipple as cap for a 50-c.c. vaccine bottle. The nipple is placed as shown, and the free edge is pulled outward and downward around the neck of the bottle.

of the salt solution from one of the 10-c.c. salt-tubes is transferred by means of a sterile capillary pipet to each of the culture-tubes, and the growth thoroughly rubbed up with a stiff platinum wire or a glass rod whose tip is bent at right angles. The suspension from the different tubes, usually amounting to about 10 c.c., is then collected in one large tube (about 150 x 19 mm.). The upper part of the tube is drawn out in the flame of a blast-lamp or Bunsen burner, as indicated in Figure 301, *B*, a short section of glass tubing being fused to the rim of the tube to serve as a handle. It is then stood aside, and when cool the narrow portion is sealed off.

The resulting hermetically sealed capsule is next thoroughly shaken for ten to twenty minutes to break up all clumps of bacteria. A few small sterile glass beads may be introduced to assist in this, but with many organisms it is not necessary.

Instead of the capsule described above, an ordinary sterile vaccine bottle may be used for the bacterial suspension. This is then covered with a rubber cap.

4. Sterilization.—The capsule is placed in a water-bath at 60° C. for forty-five minutes. This can be done in an ordinary rice-cooker, with double lid, through which a thermometer is inserted. When both compartments are filled with water it is an easy matter

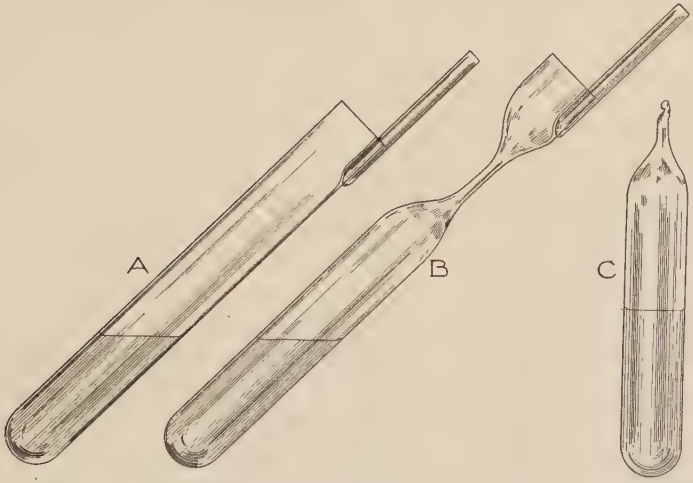


FIG. 301.—Process of making hermetically sealed capsules containing liquid: *A*, A short section of glass tubing has been fused to the top of the tube to serve as a handle; *B*, the upper part of the tube has been drawn out in a flame; *C*, the top of the capsule has been sealed.

to maintain a uniform temperature by occasional application of a small flame. The time and temperature are important: too little heat will fail to kill the bacteria, and too much will destroy the efficiency of the vaccine.

When sterilization is complete the capsule is opened, a few drops are planted on agar or blood-serum, and the capsule is again sealed.

5. Counting.—When incubation of the planted tube has shown the suspension to be sterile it is ready for counting. Some prefer to do the counting as soon as the suspension is made and before sterilization. Of the methods given here the second is the most accurate, but the third will generally be found most convenient.

Wright's Method.—There must be ready a number of clean slides, a few drops of physiologic saline on a slide or in a watch-glass; a blood-lancet, which can be improvised from a spicule of glass or a pen; and two capillary pipets with squarely broken off tips and wax-pencil marks about 2 cm. from the tip (Fig. 302). These are easily made by drawing out a piece of glass tubing, as described on page 642.

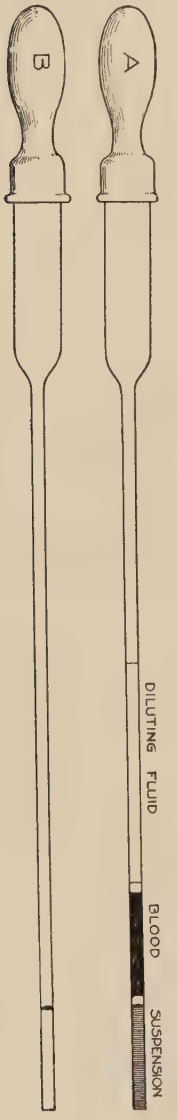


FIG. 302.—Capillary pipets: *A*, Filled for counting a vaccine by Wright's method; *B*, empty, showing wax-pencil mark. The slender portion should be narrower than here represented.

It is necessary to work quickly. After thorough shaking, the capsule is opened and a few drops forced out upon a slide. Any remaining clumps of bacteria are broken up with one of the pipets by holding it against and at right angles to the slide, and alternately sucking the fluid in and forcing it out. The pipet is most easily controlled if held in the whole hand with the rubber bulb between the thumb and the side of the index-finger. A finger is then pricked until a drop of blood appears; and into the second pipet are quickly drawn successively: 1 or 2 volumes physiologic salt solution (or, better, a 1 per cent. solution of sodium citrate, which prevents coagulation); a small bubble of air; 1 volume of blood; a small bubble of air; and, finally, 1 volume of bacterial suspension. (A "volume" is measured by the distance from the tip of the pipet to the wax-pencil mark.) The contents of the pipet are then forced out upon a slide and thoroughly mixed by sucking in and out, care being taken to avoid bubbles; after which the fluid is distributed to a number of slides and spread as in making blood-smears.

The films are stained with Wright's blood-stain or, better, by a few

minutes' application of carbol-thionin, after fixing for a minute in 1 per cent. mercuric chlorid solution. With an oil-immersion lens both the red cells and the bacteria in a number of microscopic fields are counted. The exact number is not important; for convenience 500 red cells may be counted. From the ratio between the number of bacteria and of red cells it is easy to calculate the number of bacteria in 1 c.c. of the suspension, it being known that there are 5000 million red corpuscles in a cubic centimeter of normal human blood. If there were twice as many bacteria as red corpuscles in the fields counted, the suspension would contain 1000 million bacteria per cubic centimeter.

Hemacytometer Method.—This is carried out in the same manner as a blood-count, using any convenient dilution, usually 1 : 200. A weak carbolfuchsin or gentian-violet solution, freshly filtered, may be used as diluting fluid, but the following solution, recommended by Callison, is better:

Hydrochloric acid	2 c.c.
Mercuric chlorid (0.2 per cent. solution)	100 "
Acid fuchsin (1 per cent. aqueous solution), to color.	

The color should be just deep enough not to obscure the ruled lines.

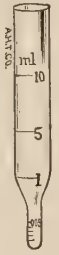


FIG. 303.—Hopkin's centrifuge tube. Useful for approximate count of bacteria in vaccines. The narrow portion at the bottom is graduated in hundredths of a cubic centimeter.

A very thin cover-glass must be used; and, after filling, the counting-chamber must be set aside for an hour or more to allow the bacteria to settle. Mallory and Wright advise the use of the shallow Helber chamber made for counting blood-plates, but many 2-mm. oil-immersion objectives have sufficient working distance to allow the use of the regular counting-chamber, provided a very thin cover is used. The heavy cover with central excavation is recommended.

Hopkin's Method.—In this the number of bacteria is estimated from their bulk after centrifugation. The bacterial suspension is filtered through a small filter of sterile cotton to remove large clumps of bacteria and bits of agar. It is then placed in a special tube (Fig. 303) and centrifugalized for half an hour at about 2800 revolutions per minute. The tube may be covered with a sterile rubber cap, or plugged with sterile cotton held in place by adhesive tape. The high speed required necessitates a powerful centrifuge. It is too great for the ordinary medical centrifuge.

During centrifugation the bacteria collect in the narrow portion, which is graduated in hundredths of a cubic centimeter. The super-

natant fluid and all of the bacterial sediment above the 0.05 mark are then removed with a sterile capillary pipet, and sterile salt solution containing 0.5 per cent. phenol is added to the 5 c.c. mark. The bacteria are then well mixed with the fluid by means of a capillary pipet. This gives a 1 per cent. bacterial suspension. In case the bacterial sediment does not reach the 0.05 mark a correspondingly smaller amount of the salt solution is added.

The number of bacteria in a 1 per cent. suspension differs with the species as follows:

	Million in each cubic centimeter.
Staphylococcus albus and aureus	10,000
Streptococcus hemolyticus	8,000
Gonococcus	8,000
Pneumococcus	2,500
Bacillus typhosus	8,000
Bacillus coli	4,000

6. Diluting.—The amount of the suspension which, when diluted to 10 c.c., will give the strength desired for the finished vaccine having been determined, this amount of salt solution is withdrawn with a hypodermic syringe from one of the bottles already prepared, and is replaced with an equal amount of suspension. Two-tenths cubic centimeter of 25 per cent. phenol in water with just enough alcohol to maintain solution is then added with a tuberculin syringe and the vaccine is ready for use. The usual strengths are: staphylococcus, 1000 million in 1 c.c., most other bacteria, 100 million in 1 c.c. Vaccines should be kept in the ice-box, and, as a rule, should not be used when more than four months old.

II. METHOD OF USE

Vaccines are administered subcutaneously, usually in the arm or abdominal wall or between the shoulder-blades. The technic is the same as for an ordinary hypodermic injection. The syringe is usually sterilized by boiling. The site of the injection may be mopped with alcohol, or may be touched with a pledget of cotton saturated with tincture of iodin or liquor cresolis compositus. The rubber cap of the container is sterilized by applying alcohol for some minutes, usually while the syringe is being sterilized, or simply placing a drop of liquor cresolis compositus upon it. The bottle is then inverted and well shaken, when the needle is plunged through the rubber and the desired quantity withdrawn. The hole seals

itself. A satisfactory syringe is the comparatively inexpensive Luer 1 c.c. "Tuberculin" syringe graduated in hundredths of a cubic centimeter.

III. DOSAGE

Owing to variations, both in virulence of organisms and susceptibility of patients, no definite dosage can be assigned. Each patient is a separate problem. Wright's original proposal was to regulate the size and frequency of dose by its effect upon the opsonic index, but this is beyond the reach of the practitioner, and is now very rarely resorted to by any one. The "clinical method" consists in beginning with a very small dose and cautiously increasing until the patient shows either improvement or some sign of a "reaction," indicated by headache, malaise, fever, exacerbation of local disease, or inflammatory reaction at the site of injection. The reaction indicates that the dose has been too large. The beginning dose of staphylococcus is about 50 million; the maximum, 1000 million or more. Of most other organisms the beginning dose is 5 million to 10 million; maximum, about 100 million. Ordinarily, injections are given once or twice a week; very small doses may be given every other day.

IV. THERAPEUTIC INDICATIONS

The therapeutic effect of vaccines depends upon their power to produce active immunity: they stimulate the production of opsonins and other antibacterial substances which enable the body to combat the infecting bacteria. Their especial field is the treatment of subacute and chronic localized infections, in some of which they offer the most effective means of treatment at our command. In most chronic infections the circulation of blood and lymph through the diseased area is very sluggish, so that the antibodies, when formed, cannot readily reach the seat of disease. Ordinary measures which favor circulation in the diseased part should, therefore, accompany the vaccine treatment Among these may be mentioned incision and drainage of abscesses, dry cupping, application of heat, Bier's hyperemia, and so forth, but such measures should not be applied during the twenty-four hours succeeding an injection, since the first effect of the vaccine may be a temporary lowering of resistance. Vaccines are of little value, and, in general, are contraindicated in very acute infections, particularly in those which are accompanied by much systemic intoxication,

for in such cases the power of the tissues to produce antibodies is already taxed to the limit. It is true, nevertheless, that remarkably beneficial results have occasionally followed their use in such acute conditions as malignant endocarditis, but here they should be tried with extreme caution.

Probably best results are obtained in staphylococcus infections, although pneumococcus, streptococcus, and colon bacillus infections sometimes respond nicely. Among clinical conditions which have been treated successfully with vaccines are furunculosis, acne vulgaris, infected operation wounds, pyelitis, cystitis, subacute otitis media, chronic gonorrhea and gonorrheal rheumatism, chronic bronchitis, bronchial asthma of bacterial origin, infections of nasal accessory sinuses, and so forth. Vaccine treatment of the mixed infection is doubtless an important aid in tuberculosis therapy, and in occasional cases the result is brilliant. When, as is common, several organisms are present in the sputum, a vaccine is made from each, except the tubercle bacillus, of which autogeneous vaccines are not used in practice. Sometimes the principal organisms are put into a single vaccine in approximately the proportions in which their colonies appeared in the primary cultures. To avoid the delay and consequent loss of virulence entailed by study and isolation of the several varieties, many workers make the bacterial suspension directly from the primary cultures. The resulting vaccines contain all strains which are present in the sputum in approximately the same relative numbers. Although open to criticism from a scientific standpoint, this method offers decided practical advantages in some cases.

V. PROPHYLACTIC USE OF VACCINES

It has been shown that vaccines are useful in preventing as well as curing infections. Their value has been especially demonstrated in typhoid fever. Three doses of about 500 million, 1000 million, and 1000 million typhoid bacilli, respectively, are given about seven to ten days apart. Results in the army, where the plan has been tried on a large scale, show that such vaccination is effective, protecting the individual for one to two years, or longer. At present a triple vaccine, containing 1000 million typhoid bacilli, 750 million paratyphoid A, and 750 million paratyphoid B in 1 c.c., is used in the army. The first dose is 0.5 c.c., the second and third 1 c.c.

VI. TUBERCULINS

Tuberculins contain the products of tubercle bacilli or their ground-up bodies, the latter class being practically vaccines. They are undoubtedly of great value in the treatment of localized tuberculosis, particularly of bones, joints, and glands; and are of rather indefinite though certainly real value in chronic pulmonary tuberculosis, especially when the disease is quiescent. The best known are Koch's old tuberculin (O. T.), bouillon filtrate (B. F.), triturate residue (T. R.), bacillary emulsion (B. E.), and purified tuberculin (Endotin). There seems to be little difference in the actions of these, although theoretically T. R. should immunize against the bacillus and B. F. against its toxic products. The choice of tuberculin is much less important than the method of administration. The making of autogenous tuberculins is impracticable, hence stock preparations are used in practice. Very recently Dryer has prepared a "defatted" tuberculin which appears to be a decided improvement over any of the others.

Since the dose is exceedingly minute, the tuberculin as purchased must be greatly diluted before it is available for use. A convenient plan is to use the rubber-capped bottles of sterile salt solution with phenol described for vaccines (p. 675), adding sufficient tuberculin to give the desired strength. The practitioner should bear in mind that while tuberculin is capable of good, it is also capable of great harm. Everything depends upon the dosage and plan of treatment. Probably a safe beginning dose for a pulmonary case is 0.00001 mg.; for gland and bone cases, about 0.0001 mg. The intervals are about one week or, rarely, three days, when very small doses are given. The dose is increased steadily, but with *extreme caution;* and should be diminished or temporarily omitted at the first indication of a "reaction," of which, in general, there are three forms:

(*a*) *General.*—Elevation of temperature (often slight), headache, malaise.

(*b*) *Local.*—Increase of local symptoms, amount of sputum, and so forth.

(*c*) *Stick.*—Inflammatory reaction at site of injection.

Treatment is usually continued until a maximum dose of 10 mg. is reached, the course extending over a year or more.

VII. TUBERCULIN IN DIAGNOSIS

The tissues of a tuberculous person are sensitized toward tuberculin, and a reaction (see preceding section) occurs when any but the most minute quantity of tuberculin is introduced into the body. Non-tuberculous persons exhibit no such reaction. This is utilized in the diagnosis of obscure forms of tuberculosis, the test being applied in a number of ways. These tests have very great diagnostic value in children, especially those under three years of age, but are often misleading in adults, positive reactions occurring in many apparently healthy individuals. Negative tests are very helpful in deciding against the existence of tuberculosis.

1. **Hypodermic Injection.**—After first determining the patient's normal temperature variations, Koch's old tuberculin is used in successive doses, three or four days apart, of 0.01, 0.1, 1, 2, 5, and 10 mg. A negative result with the largest amount is considered final. The reaction is manifested by fever within eight to twenty hours after the injection. A rise in temperature of 1° F. is generally accepted as positive. The method involves some danger of lighting up a latent process, and has been largely displaced by safer, although perhaps less reliable, methods.

2. **Calmette's Ophthalmo-reaction.**—One or 2 drops of 0.5 per cent. purified old tuberculin are instilled into one eye. Tuberculin ready prepared for this purpose is on the market. If tuberculosis exists anywhere in the body, conjunctivitis is induced within twelve to twenty-four hours. This generally subsides within a few days. The method is now rarely used since it is not without some, though slight, risk of injury to the eye. It is absolutely contraindicated in the presence of any form of ocular disease. A second instillation should not be tried in the same eye.

3. **Moro Reaction.**—A 50 per cent. ointment of old tuberculin in lanolin is rubbed into the skin of the abdomen, a piece about the size of a pea being required. Dermatitis, which appears in twenty-four to forty-eight hours, indicates a positive reaction. The ointment can be purchased ready for use.

4. **Von Pirquet's Method.**—This is the most widely used of the tuberculin tests. Two small drops of old tuberculin are placed on the skin of the front of the forearm, about 2 inches apart, and the skin is slightly scarified, first at a point midway between them, and then through each of the drops. A convenient scarifier is a

piece of heavy platinum wire, the end of which is hammered to a chisel-edge. A wooden toothpick with a chisel-shaped end is also convenient. This is held at right angles to the skin, and rotated six to twelve times with just sufficient pressure to remove the epidermis without drawing blood. In about ten minutes the excess of tuberculin is gently wiped away with cotton. No bandage is necessary. A positive reaction is shown by the appearance in twenty-four to forty-eight hours of a papule with red areola, which contrasts markedly with the small red spot left by the control scarification.

5. **Mantoux Intracutaneous Test.**—A more accurate skin test is that proposed by Mantoux. A 0.05 per cent. solution of old tuberculin in 0.5 per cent. phenol is used; also a control is made using 0.5 per cent. phenol. The forearm is cleaned with alcohol, and 0.1 c.c. of the control solution is injected *intracutaneously* with a tuberculin syringe. In like manner, 0.1 c.c. of the 0.05 per cent. old tuberculin is injected a few inches above the control. The reaction is read in twenty-four hours, or, preferably, in forty-eight hours. No reaction is considered positive unless the area of hyperemia around the tuberculin is 5 mm. greater than the control.

VIII. SCHICK TEST FOR IMMUNITY TO DIPHTHERIA

By means of this test, which was introduced by Schick in 1913, it is possible to select from a group of individuals those who are immune to diphtheria by virtue of natural or artificial immunity. In an epidemic of diphtheria, therefore, it may be of very great value as a means of determining who shall and who shall not receive prophylactic injections of antitoxin. The reaction is not applicable to the diagnosis of diphtheria.

The test consists in the intradermal injection of 0.2 c.c. of diphtheria toxin so diluted that this amount contains one-fiftieth the minimum lethal dose for the guinea-pig. The dilution should be freshly made and must not be used after twelve hours. The toxin may be obtained from the biologic supply houses in packages containing a small amount of toxin and sufficient sterile saline to make the required dilution when the two are mixed. A small all-glass tuberculin syringe with short No. 26 needle is used.

To make the test, clean an area on the skin of the arm or forearm with a pledget of cotton and alcohol, encircle the arm with

the thumb and index-finger, drawing the skin tense between them. Inject 0.2 c.c. of the freshly diluted toxin *into* the skin, not through it. A definite white wheal-like swelling appears if the injection is rightly made. A like amount of the diluted toxin which has been heated to 75° C. for ten minutes to destroy the toxin is injected into the other arm as a control. It is customary to use the right arm for the test and the left arm for the control.

No reaction should occur in those who are immune to diphtheria. In those who are not immune a distinct red spot about 1 to 2 cm. in diameter appears at the site of injection of the unheated toxin within twenty-four to thirty-six hours. This is followed by induration, reaching its height on or about the fourth day. The redness and induration gradually disappear, leaving a brownish area of pigmentation which sometimes persists for three or four months. No such reaction occurs at the site of the control injection. Sometimes there is a reaction at both sites due to bacillary proteins which are not destroyed by heating, as is toxin. In such cases, if the test reaction is much greater than the control, the test may be considered positive and the patient regarded as susceptible to diphtheria.

The Schick test is very reliable if a proper toxin be used and if the reaction be correctly interpreted. Among 524 individuals tested, Moody found 45.2 per cent. of positives. The percentage is higher in children and lower in adults.

IX. DICK TEST FOR IMMUNITY TO SCARLET FEVER

A test which appears to bear the same relation to scarlet fever that the Schick test bears to diphtheria has been devised by George F. and Gladys H. Dick. This consists in injecting, intradermally, 0.1 c.c. of 1 : 1000 dilution of the Berkefeld filtrate of a culture of a streptococcus which has been isolated from a case of scarlet fever and which is capable of producing the disease in human beings. This filtrate can now be purchased, ready for use, from the biological supply houses.

The reactions are observed at the end of twenty-four hours, and are classified as *negative* when there is no more than a faint pink streak along the course of the needle; as *slightly positive* when there is a faint red area less than 1 cm. in diameter with no swelling; as *positive* when the area is from 1 to 3 cm. in diameter, bright red,

with some swelling of the skin; as *strongly positive* when the area is intensely red, from 3 to 5 cm. in diameter, and markedly swollen, with sharply raised edge.

Positive and strongly positive reactions may be interpreted as indicating susceptibility to scarlet fever; negative reactions as indicating immunity.

X. TESTS FOR HYPERSUSCEPTIBILITY

Hypersusceptibility to various organic substances has of recent years come to be recognized as playing an important rôle in medicine. To it are ascribed such conditions as hay-fever, asthma, shock following administration of antitoxins and therapeutic sera, the urticarias, and diarrheas which in some persons follow ingestion of certain foods. It is unnecessary to discuss the nature of the phenomena or the application of the special terms—allergy, anaphylaxis, and idiosyncrasy.

Hypersusceptibility to any particular substance—pollen of certain plants, dandruff or serum of animals, drugs, articles of food—can be recognized by a simple skin test. This consists in applying an extract of the substance to a slight incision in the skin made with the point of a sharp knife only to such a depth as to introduce the substance below the epidermis, but drawing no blood.

A more accurate method is to inject *intradermally* definite amounts of the protein substance. The amount to be used may vary with the individual. In general, it is often the practice to inject 0.05 c.c. of an extract so diluted that 1 c.c. contains 0.001 mg. of the protein. Care should be taken to avoid injecting *under* the skin, otherwise unpleasant and even dangerous symptoms might be induced in an extremely sensitive individual.

Substances such as dandruff and pollens are extracted with decinormal sodium hydroxid solution, which is then used for the test, or the substance may be placed on the incision in the skin with a drop of the decinormal solution and very gently mixed and rubbed into the incision. Extracts of a long list of pollens, dandruff, and other substances, especially prepared for the tests, are available commercially. Drugs may be used in 10 per cent. solution. In the case of antitoxin and the various therapeutic sera it is not the immune bodies which cause the dangerous reaction, but rather the horse-serum with which they are administered. Hypersensi-

tiveness to horse-serum is best recognized by intradermal injection of 0.2 c.c. of 1 : 10 dilution of normal horse-serum in saline solution.

The arm is the usual site for the test, and a control test is made upon the other arm with omission of the specific substance. Tests with different substances may be carried out simultaneously. A positive reaction, indicating hypersusceptibility to the particular substance used in the test, is manifested by an urticarial wheal surrounded by a broad zone of redness. This appears within half an hour, often within five minutes, and begins to fade in an hour or two.

APPENDIX

I. OFFICE LABORATORY METHODS AND EQUIPMENT

It is not to be expected that a physician in active practice will make routine use of all the methods described in this book. Although he will need nearly all of them for the study of his more difficult cases, his daily laboratory work will probably be limited to a few simple procedures. With this in mind, the following list of laboratory procedures is suggested as the minimum with which a physician should be thoroughly familiar and upon which he may build as his practice requires. The methods are selected because of their simplicity and practical usefulness:

METHODS FOR OFFICE ROUTINE

Sputum

Careful inspection (p. 51).
Simple microscopic examination unstained (p. 54).
Examination for tubercle bacilli (p. 64).

Urine

Reaction (p. 89).
Specific gravity (p. 91).
Calculation of total solids (p. 93).
Phenolsulphonephthalein and Mosenthal's tests of kidney function (p. 94).
Albumin, qualitative:
    Roberts' ring test (p. 136).
    Purdy's heat test (p. 136).
Albumin, quantitative:
    Tsuchiya's method (p. 138).
Sugar, qualitative:
    Benedict's test (p. 143).
Sugar, quantitative:
    Benedict's method (p. 147), or Einhorn's yeast method (p. 148).
Acetone, Lange's or Rothera's test (p. 154).
Diacetic acid, Gerhardt's test (p. 155).
Bile, Gmelin's test (p. 157).
Indican, Obermayer's test (p. 119).
Microscopic examination (p. 170).

Blood

Coagulation time, any simple method (p. 226).
Bleeding time (p. 231).
Hemoglobin, Dare or Sahli method (pp. 237–239).
Red corpuscle count (p. 241).
Color index calculation (p. 251).
Leukocyte count (p. 261).
Differential leukocyte count (p. 284).
Microscopic examination of stained films for pathologic red cells and malarial parasites (pp. 276, 314).

Stomach Contents

Careful inspection (p. 396).
Total acidity, Töpfer's method (p. 402).
Free hydrochloric acid, Töpfer's method (p. 404).
Lactic acid, Kelling's test (p. 399).
Microscopic examination (p. 406).

Feces

Careful inspection (p. 419).
Occult blood, benzidin test after extraction with ether (p. 423).
Microscopic examination:
(*a*) for parasites or their ova (p. 437).
(*b*) to ascertain state of digestion (p. 441).

Serum Methods

Widal test by macroscopic method, using one of the commercial outfits (p. 575).

Miscellaneous

Microscopic examination of pus:
(*a*) Simple stain (p. 657).
(*b*) Gram's method (p. 657).
Puncture fluids:
(*a*) Careful inspection (pp. 526 and 530).
(*b*) Microscopic examination for bacteria and differential cell count (p. 526).
Syphilitic material for spirochetes, Giemsa's or Wright's stain or India-ink method (p. 558).
Milk:
Fat, Leffmann-Beam method (p. 554).
Protein, by calculation (p. 553).

EQUIPMENT

A list of equipment which is sufficient for all the above-mentioned methods (and for many others in addition) is given below. The total cost, exclusive of the furniture, but including a first-class

microscope and mechanical stage, will be about $225 to $250.[1] There is no real economy in purchasing instruments of inferior quality.

A. FURNITURE

A table, with drawer, and a few shelves for bottles and glassware constitute the only really essential laboratory furniture even for fairly extensive work. When a special room is not available these may stand behind a screen in the physician's consulting room. Gas and running water are very desirable, but not absolutely necessary.

The shelves may conveniently take the form of a shallow case without doors, which stands upon the back of the table and which, in addition to the shelves, has two tall compartments, one for the combined buret and filter stand, the other for the microscope in its case. If space allows, however, it will be found more satisfactory to keep the microscope under a glass bell-jar (or pasteboard cover, p. 37) on a stand or small table before a window. It is thus always ready for use and is away from the neighborhood of corroding chemicals. A stool or chair of the proper height (p. 34) should be at hand.

A convenient reservoir for wash-water is a large bottle, which stands upon the top shelf and from which water is siphoned by means of a rubber tube with a medicine-dropper tip and a Mohr pinch-cock. The glass tip should hang directly over a miniature sink consisting of a large glass funnel whose stem passes through the table top and drains by means of a rubber tube into an earthen jar below. All staining should be done over this funnel-sink, the slides being supported upon a rack consisting essentially of two small rods about 2 inches apart placed across the top of the funnel.

The following **acid-proof wood finish** is extensively used for table tops in the laboratories of this country. It gives an ebony-black surface which resists practically all reagents. The wood must be new, or at least not painted, varnished, or waxed.

[1] The entire outfit, with ready prepared reagents and staining solutions, can be purchased of Denver Fire Clay Co., 1742 Champa St., Denver; Paul Weiss, 1620 Arapahoe St., Denver; A. H. Thomas Co., West Washington Square, Philadelphia, and probably many other supply houses.

Solution No. 1:

Copper sulphate	125 gm.
Potassium chlorate (or permanganate)	125 "
Water	1000 c.c.

Solution No. 2:

Anilin oil	120 c.c.
Hydrochloric acid, concentrated	180 "
Water	1000 "

Apply two coats of Solution No. 1, hot, and then two coats of Solution No. 2, without heating, allowing each coat to dry thoroughly before the next is applied. When the last coat is dry remove the excess of the chemicals by rubbing with a coarse cloth. Finally rub thoroughly with a mixture of equal parts of turpentine and linseed oil.

B. APPARATUS

1 Basin of white enameled ware.

2 Beakers with lip, about 50-c.c. capacity, preferably of Pyrex glass.

1 Blood-lancet or some substitute, as a No. 2 Hagedorn needle (p. 221).

1 Bunsen burner with rubber tubing, the small "micro" burner being especially satisfactory. An alcohol lamp will answer.

1 Buret, 25-c.c. capacity, preferably with Schellbach stripe. An accurate 10-c.c. graduated pipet may be used for much work, but is not so satisfactory as the buret.

1 Centrifuge, hand, electric or water power (Figs. 40, 41). The last is cheap and satisfactory. Metal shields with flat bottoms and rubber cushions are preferable to the ordinary conical aluminum shields because they allow the use of ordinary test-tubes as well as conical centrifuge tubes.

1 Esbach tube (Fig. 47).

1 Pack filter-papers, round, about 15 cm. in diameter, good quality.

1 Funnel, glass, about 10 cm. in diameter.

4 Feet of glass tubing, about 7 or 8 mm. outside diameter, of soft glass for making pipets. Should be of such size that rubber nipples may be fitted.

1 Graduated cylinder, 100 c.c., double graduations. This is used chiefly for making solutions.

1 Hemacytometer. Probably the most satisfactory outfit consists of an "open" counting chamber with Neubauer ruling, a "red pipet" and a "white pipet." It should be accompanied by the certificate of the U. S. Bureau of Standards.

1 Hemoglobinometer. The Dare will probably be found most convenient if the price is not prohibitive; otherwise the Sahli is recommended. A Tallqvist book should be carried in the hand-bag.

1 Pack lens-cleaning paper. Two rows of stitching, ½ inch apart, may be run across the middle of the package on the sewing machine, and the package then cut into little booklets of convenient size.

1 Box labels for bottles. Denison's No. A-4 is a useful size.

1 Box labels for slides.

1 Mechanical stage, attachable (p. 43).

4 Medicine-droppers: two, labeled "Stain" and "Water" respectively, to be reserved for use with Wright's blood-stain; one, which delivers the proper sized drop, to be reserved for the quantitative sugar estimation (p. 147).

1 Eye-piece micrometer. The card-board micrometer made as described on page 39 will answer for most clinical work.

1 Microscope equipped as described on page 42.

50 Micro cover-glasses, No. 2 thickness. The 22 mm. squares are most convenient for general purposes.

1 Box (½ gross) micro slides, 75 x 25 mm., clear white glass, medium thickness, ground edges.

1 Pencil, wax, for writing on glass, red or blue.

1 Petri dish with cover, about 15 cm. in diameter.

1 Pipet, 10 c.c., graduated.

1 Rule, celluloid, 6 inches and 15 cm. These are sold by Bausch and Lomb Optical Co., and Spencer Lens Co.

1 Stand for filter, buret, and so forth.

1 Stomach-tube. The Rehfuss type is required if fractional method of examination is employed and is best for all purposes.

1 Test-glass, conical. A wine-glass will serve.

12 Test-tubes, size about 125 x 16 mm., without flange.

1 Test-tube brush, bristle, with tuft at tip.

1 Test-tube rack holding 6 tubes.

1 Urinometer with cylinder. Must have wide graduations. Test with distilled water.

1 Widal test outfit, macroscopic method.

1 Box wooden toothpicks.

C. REAGENTS AND STAINS

All staining solutions and many reagents are best kept in small dropping bottles, of which the "flat-topped T. K." pattern is most satisfactory. Other reagents may be kept in ordinary round pre-

scription bottles of 4- to 8-ounces capacity. Bottles containing highly volatile reagents should be sealed with paraffin if not in constant use; while those containing strong caustic soda solutions should have rubber stoppers.

Most staining solutions and chemical reagents can be purchased ready prepared. For the physician who does only a small amount of work the "Soloid" tablets manufactured by Burroughs, Wellcome & Co. are convenient and satisfactory. Similar tablets are now made in this country. These tablets have only to be dissolved in a specified amount of the appropriate fluid to produce the finished solution. Most of the stains and many of the commoner reagents are supplied in this form.

If, however, his time permits the physician will find it more satisfactory and much more economical to prepare his own solutions, with exception of normal solutions and a very few stains.

Reagents

50 c.c. Acid, acetic, glacial, 99½ per cent. Other strengths can be made from this as desired.

50 c.c. Acid, hydrochloric, C.P., sp. gr. 1.16. Contains about 32 per cent. HCl. An approximate decinormal solution for use with the Sahli hemoglobinometer can be made by adding 12 c.c. of this acid to 988 c.c. distilled water.

50 c.c. Acid, nitric, C.P. Yellow nitric acid can be made from this by adding a splinter of pine (match stick) or allowing it to stand in the sunlight for a short time.

50 c.c. Acid, sulphuric, C.P.

50 c.c. Alcohol, amylic, C.P. Used in the estimation of fat in milk.

200 c.c. Alcohol, ethylic (grain alcohol). This is ordinarily about 93 to 95 per cent., and other strengths can be made as desired. Whenever the word "alcohol" is used in the text without qualification, this alcohol is meant. The following is a simple rule for diluting alcohol to any desired strength: Take of alcohol a number of parts equal to the percentage desired and add of water a number of parts equal to the difference between the desired strength and the strength diluted. For example, to dilute 80 per cent. alcohol to 50 per cent., take 50 c.c. of the alcohol and add 80–50 = 30 c.c. of water.

100 c.c. Alcohol, methylic, Merck's "Reagent," for making Wright's blood-stain. May be omitted if the stain is purchased ready prepared.

100 c.c. Ammonium hydroxid (strong ammonia), sp. gr. 0.9.

200 c.c. Benedict's solution for qualitative sugar test (p. 144).

100 c.c. Benedict's solution for quantitative sugar estimation (p. 148).

10 gm. benzidin. Specify "for blood test."

1 tube Canada balsam in xylol. Necessary only if permanent mounts are to be made.

100 c.c. Chloroform, U. S. P.

100 c.c. Diluting fluid for red corpuscle count, Hayem's preferred (p. 250).

100 c.c. Diluting fluid for leukocyte count (p. 264).

30 c.c. Dimethyl-amino-azo-benzol, 0.5 per cent. alcoholic solution.

100 c.c. Tsuchiya's solution (p. 138).

200 c.c. Ether, sulphuric, U. S. P.

30 c.c. Ferric chlorid, 10 per cent. aqueous solution.

100 c.c. Formalin (40 per cent. solution of formaldehyd gas). The expression "10 per cent. formalin" means 1 part of this 40 per cent. solution and 9 parts of water, making a 4 per cent. solution of formaldehyd gas.

50 c.c. Hydrogen peroxid, U. S. P.

1 Vial litmus paper, Squibb, red.

1 Vial litmus paper, Squibb, blue.

25 c.c. Lugol's solution (*Liquor Iodi Compositus*, U. S. P.). Gram's iodin solution (p. 698) can be made from this by adding fourteen times its volume of water.

50 gm. Magnesium sulphate, C.P., for making Roberts' solution for albumin in urine.

100 c.c. Obermayer's reagent for indican (p. 119).

25 c.c. Oil of cedar for immersion. A sufficient quantity is usually supplied with the microscope when purchased.

25 c.c. Phenolphthalein, 1 or 0.5 per cent. solution in alcohol.

10 Ampules of phenolsulphonephthalein.

50 gm. Sodium chlorid, C.P., for Purdy's albumin test. Table salt may be used, but is not so good.

1000 c.c. Sodium hydroxid, decinormal solution. The practitioner will find it best to purchase this solution ready prepared. Most chemical supply houses carry it in stock. For rough clinical work 4.1 grams of Merck's "Sodium hydrate by alcohol" from a freshly opened bottle may be dissolved in 100 c.c. distilled water. This makes a normal solution and must be diluted with 9 volumes of water to make the decinormal solution. The preparation of accurate normal solutions is described on pages 700, 701.

25 gm. Sodium nitroprussid, C.P., crystals.

50 gm. Talc, purified (*Talcum purificatum*, U. S. P.), or diatomaceous earth (*Kieselguhr*) for clearing urine.

2000 c.c. Water, distilled. In some regions ordinary tap-water answers for practically all purposes.

Stains

It will be found most satisfactory to have on hand a stock of dry stains (which keep well) and to make solutions as needed. Ordinarily the smallest quantity obtainable in an unbroken package should be purchased. The most reliable stains now bear a stamp on the bottle, showing that they have been approved by the Commission on Standardization of Biological Stains. This committee is composed of representatives of the leading societies in the United States interested in bacteriology, botany, dye chemistry, pathology, and zoölogy. The following dry stains make up a fairly complete stock for the clinical laboratory: Fuchsin, basic; crystal violet; methylene-blue, B.X.; methyl-green; pyronin, and Wright's stain. Wright's stain is obtainable in 1-gm. vials, the others in 10-gm. vials. The most frequently used solutions which can be purchased in 25-c.c. bottles are:

Carbolfuchsin (p. 697).

Carbol-gentian-violet (p. 697).

Giemsa's stain (p. 275). This is not necessary if Wright's stain or the India-ink method be used for spirochetes.

Löffler's alkaline methylene-blue (p. 698).

Pappenheim's methylene-blue contrast stain for tubercle bacilli (p. 698).

Pappenheim's pyronin-methyl-green stain (p. 698).

Wright's blood-stain (p. 272). Much of the solution on the market is unsatisfactory.

II. STAINING SOLUTIONS

In this section are given the formulæ for staining fluids which have general use, particularly for identification of bacteria. Blood-stains and others which are used only for special purposes are discussed in the body of the book and may be found by consulting the Index.

1. **Carbol Thionin.**—Saturated solution thionin in 50 per cent. alcohol, 20 c.c.; 2 per cent. aqueous solution phenol, 100 c.c.

This stain is especially useful in counting bacteria for standardization of vaccines (p. 679). It can be used as a general stain. In blood work it is sometimes used for the malarial parasite and for demonstration of basophilic degeneration of the red cells. The fluid is applied for one-half to three minutes, after fixation by heat,

or about a minute in 1 per cent. aqueous solution of mercuric chlorid or 1 per cent. formalin in alcohol.

2. **Crystal violet** is a powerful bacterial stain which may be substituted for gentian violet in all formulas and is more satisfactory. A solution of 2 gm. crystal violet in 100 c.c. methyl alcohol of the highest purity is probably the best stain for Gram's method.

3. **Fuchsin.**—This dye should not be confused with acid fuchsin. Its solutions are generally made with phenol as a mordant, and they are then very powerful bacterial stains, with a strong tendency to overstaining. They are used chiefly for the tubercle bacillus.

Czaplewski's carbolfuchsin is superior to the widely used Ziehl solution in that it acts more quickly and is permanent. To 1 gm. fuchsin and 5 c.c. liquefied phenol add 50 c.c. glycerol, with constant stirring; and finally add 50 c.c. water, mix well, and filter.

4. **Gentian Violet.**—This dye has long been widely used as a bacterial stain, especially for Gram's method, but is now being rapidly displaced by crystal violet and methyl violet. The combinations given below may be used interchangeably, but the solution with phenol is probably most serviceable. Formalin-gentian-violet remains good for years, but is less satisfactory for Gram's method than the others because it is not readily decolorized by alcohol.

Methyl violet, or crystal violet, may be substituted for gentian violet in these formulæ, and either is preferable to it.

Anilin-gentian Violet.—Ehrlich's formula is the one generally used, but this keeps only a few weeks. *Stirling's solution*, which keeps much better and seems to give equal results, is as follows: gentian violet, 5 gm.; alcohol, 10 c.c.; anilin oil, 2 cc..; water, 88 c.c.

Czaplewski's Carbol-gentian-violet.—To 1 gm. gentian violet and 5 c.c. liquefied phenol add 50 c.c. glycerol with constant stirring; finally add 50 c.c. water, mix well, and filter.

Formalin-gentian-violet consists of 5 per cent. solution formalin, 75 parts; saturated alcoholic solution gentian violet, 25 parts.

5. **Hematoxylin** is one of the best nuclear stains available. There are many combinations, most of which require weeks or months for "ripening." The following is a good solution which is ready for use as soon as made:

Harris' Hematoxylin.—Dissolve 1 gm. hematoxylin crystals in 10 c.c. alcohol. Dissolve 20 gm. ammonia alum in 200 c.c. distilled water with the aid of heat, and add the alcoholic hematoxylin solu-

tion. Bring the mixture to a boil, and add $\frac{1}{2}$ gram of mercuric oxid. As soon as the solution assumes a dark purple color, remove the vessel from the flame and cool quickly in a basin of cold water.

6. **Iodin** is used as a part of Gram's method and as a special stain for various purposes. For starch a very weak solution is desirable; for *Leptotrichia buccalis* a strong solution such as Lugol's. The solutions deteriorate upon long standing.

Gram's Iodin Solution.—Iodin, 1 gm.; potassium iodid, 2 gm.; water, 300 c.c.

Lugol's solution (*Liquor Iodi Compositus*, U. S. P.) consists of iodin, 5 gm.; potassium iodid, 10 gm.; water, 100 c.c. Gram's iodin solution may be made from this by adding fourteen times its volume of water.

7. **Methylene-blue** is a widely used basic dye which does not readily overstain. The following solutions are useful:

Gabbet's Stain.—This is used in Gabbet's method for the tubercle bacillus. It consists of methylene-blue, 2 gm.; water, 75 c.c.; concentrated sulphuric acid, 25 c.c.

Löffler's alkaline methylene-blue is one of the most useful bacterial stains for general purposes. The solution is applied at room temperature for thirty seconds to three minutes, and is followed by rinsing in water. Fixation may be by heat or chemicals. The stain is composed of 30 parts of a saturated alcoholic solution of methylene-blue and 100 parts of a 1 : 10,000 aqueous solution of potassium hydroxid. It keeps indefinitely.

Pappenheim's methylene-blue solution is used as decolorizer and contrast stain in Pappenheim's method for the tubercle bacillus. Dissolve 1 gm. corallin (rosolic acid) in 100 c.c. absolute alcohol; saturate with methylene-blue, and add 20 c.c. glycerol.

8. **Methyl violet** is a useful bacterial stain which may be advantageously substituted for gentian violet in all formulas given on page 697.

9. **Pyronin.**—Used in strong aqueous solution this is useful as a contrast stain in Gram's method, but results are more satisfactory when the dye is combined with methyl green.

Pappenheim's Pyronin-methyl-green Stain.—This solution colors bacteria red and nuclei of cells blue. It is, therefore, especially useful for intracellular bacteria like the gonococcus and the influenza bacillus. It is a good stain for routine purposes, is a most

excellent contrast stain for Gram's method, and is also used to demonstrate Döhle's inclusion bodies in the blood. It colors the cytoplasm of lymphocytes bright red, and has been used as a differential stain for these cells. The solution is applied cold for one-half to five minutes. It consists of saturated aqueous solution of methyl-green, 3 to 4 parts, and saturated aqueous solution of pyronin, 1 to $1\frac{1}{2}$ parts. It is a good plan to keep these solutions in stock and to mix a new lot of the staining fluid about once a month. If it stains too deeply with either dye, the proper balance is attained by adding a little of the other.

10. **Safranin** is widely used as a contrast stain for Gram's method, usually in 1 per cent. aqueous solution.

11. **Simple Bacterial Stains.**—A simple solution of any basic anilin dye (methylene-blue, basic fuchsin, gentian violet, and so forth) will stain nearly all bacteria. These simple solutions are not much used in the clinical laboratory, because other stains, such as Löffler's methylene-blue and Pappenheim's pyronin-methyl-green stain, which serve the purpose even better, are at hand.

12. **Sudan III** is a valuable stain for fat, to which it gives an orange color. Scharlach R is a similar but stronger dye, and may be substituted to advantage. They may be used as a saturated solution in 70 per cent. alcohol or in the following combination:

Herxheimer's sudan III consists of equal parts of 70 per cent. alcohol and acetone saturated with sudan III (or scharlach R).

III. NORMAL SOLUTIONS

A normal solution is one which contains in each liter enough of a chemical substance to replace or unite with 1 gram of hydrogen. The molecular weight of the substance divided by its valence and expressed in grams is dissolved in sufficient water to make 1 liter. In the case of a univalent substance like hydrochloric acid the molecular weight directly indicates the number of grams to the liter. Equal volumes of all normal solutions are equivalent, for example, 1 c.c. of a normal acid solution exactly combines with 1 c.c. of a normal alkali. The solutions are made up as normal solutions, and for use are generally diluted with water to one-tenth, one-twentieth, or one-fiftieth the normal strength, giving "decinormal," "twentieth normal," and "fiftieth normal" solutions.

The normal solutions most frequently used are the following:

	Grams for each liter.
Hydrochloric acid	36.46
Oxalic acid	63.03
Sulphuric acid	49.04
Potassium hydroxid	56.12
Potassium permanganate	31.63
Silver nitrate	169.97
Sodium carbonate	53.05
Sodium chlorid	58.50
Sodium hydroxid	40.06

Of the above, the only solutions ordinarily required in a small laboratory are normal sodium hydroxid and normal hydrochloric acid, from which decinormal solutions are made as needed, and only these will be described in detail. They can be purchased ready prepared from any reliable chemical supply house.

Normal Sodium Hydroxid.—Since sodium hydroxid absorbs water and unites with carbon dioxid of the air it is not of uniform strength, and accurate solutions cannot be made by weighing.

Dissolve 46 gm. of sodium hydroxid in 1100 c.c. of distilled water. This solution will be of more than normal strength and must be standardized against a normal acid solution. For this purpose oxalic acid is used because it can be weighed accurately. From a bottle of chemically pure oxalic acid select the most nearly perfect crystals, weigh out 6.3 gm. on a sensitive balance, place in a 100-c.c. volumetric flask, and make up to 100 c.c. with distilled water. Now pipet 10 c.c. of this normal oxalic acid solution into a beaker, add 3 drops of phenolphthalein indicator, and add the solution of sodium hydroxid from a buret until a faint but permanent pink color appears. If the sodium hydroxid solution were of correct strength exactly 10 c.c. would be required; but it will be too strong, and less than 10 c.c. will be used. The difference between 10 c.c. and the amount used indicates the amount of water which must be added to reduce it to the correct strength. If, for example, 9.5 c.c. were used, then 0.5 c.c. of distilled water must be added for each 9.5 c.c. of the sodium hydroxid solution. After this has been added and well mixed, refill the buret with the newly diluted solution and repeat the titration to check the accuracy of the work, using, preferably, 20 or 50 c.c. of the acid.

To make a decinormal solution add 1 volume of the normal solution to 9 volumes of distilled water.

In emergencies a decinormal solution of sufficient accuracy for rough clinical work may be made by dissolving 4.1 grams of Merck's "sodium hydroxid by alcohol" from a freshly opened bottle in 1000 c.c. of distilled water.

Normal Hydrochloric Acid.—To 135 c.c. of concentrated chemically pure hydrochloric acid add 1 liter of distilled water, and mix well. This makes a solution which is slightly too strong and must be standardized by titrating with a known normal alkali. Pipet exactly 10 c.c. of the acid solution into a beaker, add 3 drops of phenolphthalein indicator, and titrate with an accurate normal solution of sodium hydroxid until a faint but permanent pink color appears. If the acid were accurately normal, exactly 10 c.c. of the alkali would be used; but it will, in fact, be too strong and more than 10 c.c. will be required. The excess over 10 c.c. indicates the amount of water which must be added to each 10 c.c. of the acid to reduce it to correct normal strength. If, for example, 10.6 c.c. of the normal sodium hydroxid are required, then 60 c.c. of distilled water must be added to 1000 c.c. of the acid solution. After the water has been added and well mixed the titration should be repeated, using 20 c.c. of the acid.

To make decinormal hydrochloric acid add 1 volume of the normal solution to 9 volumes of water.

Approximately decinormal hydrochloric acid for use with the Sahli hemoglobinometer can be made by adding 1.2 c.c. of concentrated hydrochloric acid to 98.8 c.c. of distilled water. A few drops of chloroform are added as preservative.

IV. PHYSIOLOGIC SOLUTIONS

Physiologic solutions are so made that they contain the same percentage of various salts as are found in the fluids of the animal body.

Physiologic or "Normal saline" is made of sodium chlorid (C. P.), 0.85 gm.; distilled water, 100 c.c.

Buffered salt solution (Mason and Sanford) is described on page 593.

Locke's solution consists of sodium chlorid, 0.9 gm.; calcium

chlorid, 0.024 gm.; potassium chlorid, 0.042 gm.; sodium carbonate, 0.02 gm.; dextrose, 0.25 gm.; distilled water, 100 c.c.

Ringer's solution (modified by Porter) consists of sodium chlorid, 0.7 gm.; calcium chlorid, 0.0026 gm.; potassium chlorid, 0.035 gm.; distilled water, 100 c.c.

V. WEIGHTS AND MEASURES, WITH EQUIVALENTS

METRIC

Meter (unit of length): Millimeter (mm.) = $\frac{1}{1000}$ meter.
Centimeter (cm.) = $\frac{1}{100}$ meter.
Kilometer = 1000 meters.
Micron (μ) = $\frac{1}{1000}$ millimeter.

Gram (unit of weight): Milligram (mg.) = $\frac{1}{1000}$ gram.
Kilogram (kilo.) = 1000 grams.

Liter (unit of capacity): Cubic centimeter = $\frac{1}{1000}$ liter. = Same measure as milliliter (ml.).

1 Millimeter = { 0.03937 ($\frac{1}{25}$ approx.) in.; 1000 microns.

1 Centimeter = { 0.3937 ($\frac{2}{5}$ approx.) in.; 0.0328 feet.

1 Meter = { 39.37 in.; 3.28 feet.

1 Micron (μ) = { $\frac{1}{25000}$ in.; 0.001 millimeter.

1 Gram = { 15.43 grains.; 0.563 dram, 0.035 ounce, 0.0022 pound } Avoir.; 0.257 dram, 0.032 ounce, 0.0027 pound } Apoth.

1 Kilogram = { 35.27 ounce (Avoir.).; 2.2 pound (Avoir.).

1 Liter = { 1.056 (1 approx.) quart.; 61.02 cubic inches.; 1000 cu. centimeters.

1 Sq. Millimeter = 0.00155
1 Sq. Centimeter = 0.1550
1 Sq. Meter = 1550
} sq. in.
1 Sq. Meter = 10.76 sq. feet.

1 Cu. Millimeter = 0.00006
1 Cu. Centimeter = 0.0610
} cu. in.
1 Cu. Centimeter = 0.001 liter.

1 Cu. Meter = { 35.32 cu. feet.; 61025.4 cu. in.

1 Inch = 25.399 millimeters.
1 Foot = 30.48 centimeters.
1 Sq. Inch = 6.451 sq. centimeters.
1 Sq. Foot = 0.093 sq. meter.
1 Cu. Inch = 16.387 cu. centimeters.
1 Cu. Foot = 0.028 cu. meter.

AVOIRDUPOIS WEIGHT

1 Ounce = { 437.5 grains. / 16 drams.
1 Pound = 16 ounces.

1 Grain = 0.065 ($\frac{3}{50}$ approx.)
1 Dram = 1.77 ($1\frac{3}{4}$ approx.)
1 Ounce = 28.35 (30 approx.)
1 Pound = 453.59 (500 approx.) } grams.
1 Pound = 27.7 cu. inches.
1 Pound = 1.215 lb. Troy.

APOTHECARIES' MEASURE

1 Dram = 60 minims.
1 Ounce = 8 drams.
1 Pint = 16 ounces.
1 Gallon = 8 pints.

1 Dram = 3.70
1 Ounce = 29.57
1 Pint = 473.1
1 Gallon = 3785.4 } cu. centimeters.
1 Gallon = 231 cu. inches.

APOTHECARIES' WEIGHT

1 Scruple = 20 grains.
1 Dram = 3 scruples = 60 grains.
1 Ounce = 8 drams = 480 grains.
1 Pound = 12 ounces.

1 Grain = 0.065
1 Dram = 3.887
1 Ounce = 31.10
1 Pound = 373.2 } grams.

To convert	*minims*	into	*cubic centimeters*	multiply by	0.061
" "	*fluidounces*	"	*cubic centimeters*	"	" 29.57
" "	*grains*	"	*grams*	"	" 0.0648
" "	*drams*	"	*grams*	"	" 3.887
" "	*cubic centimeters*	"	*minims*	"	" 16.23
" "	*cubic centimeters*	"	*fluidounces*	"	" 0.0338
" "	*grams*	"	*grains*	"	" 15.432
" "	*grams*	"	*drams*	"	" 0.257

TEMPERATURE

Centigrade.	Fahrenheit.	Centigrade.	Fahrenheit.
110°	230°	37°	98.6°
100	212	36.5	97.7
95	203	36	96.8
90	194	35.5	95.9
85	185	35	95
80	176	34	93.2
75	167	33	91.4
70	158	32	89.6
65	149	31	87.8
60	140	30	86
55	131	25	77
50	122	20	68
45	113	15	59
44	111.2	10	50
43	109.4	+5	41
42	107.6	0	32
41	105.8	—5	23
40.5	104.9	—10	14
40	104	—15	+5
39.5	103.1	—20	—4
39	102.2		
38.5	101.3	0.54° =	1°
38	100.4	1 =	1.8
37.5	99.5	2 =	3.6
		2.5 =	4.5

To convert Fahrenheit into Centigrade, subtract 32 and multiply by 0.555.

To convert Centigrade into Fahrenheit, multiply by 1.8 and add 32.

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